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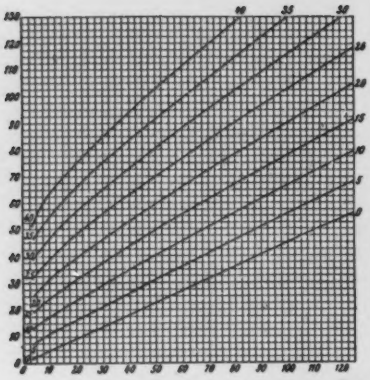

TABLES AND NOMOGRAMS OF HYDROCHEMICAL ANALYSIS

by I. Yu. Sokolov

Translated from Russian

Upon completion of analytical experiments, every research chemist, whether he uses a "test tube" or a "пробирка," must calculate his findings in the international language of figures in two forms (weights and equivalents), and often three (weights, equivalents and percent-equivalents), and then compare experimental data with theoretical values.

Technicians and statisticians working with the results of hydrochemical analyses performed at different times by different laboratories inevitably face the problem of converting their figures to one system.

All such calculations are considerably simplified by the use of the tables and nomograms in this book, originally published by the State Scientific and Technical Press for Literature on Geology and the Conservation of Mineral Resources, Moscow.

All the tables and nomograms are based on analytical results expressed in the form widely used in hydrogeological practice—milligrams per liter (weight form) and milligram-equivalents per liter (equivalent form). For calculation of percent-equivalents, the sum of cation equivalents and the sum of anion equivalents are taken as 100% each. Several new tables are presented for the first time, and the many tables for converting water-analysis results from one form to another make it possible to find the milligram-equivalents for any practically possible content of a component in water, accurate to the second decimal place, and the weight content of substances to tenths of a milligram.

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International atomic weights (Appendix)

83 pages **$4.35**

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ISOLATION AND CHEMICAL STUDY OF SEPTIC VIBRIO TOXOID

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By the use of the method of salting out with neutral salts and precipitation with acids and organic solvents it has been possible to isolate in pure form and to establish the protein nature of a number of bacterial exotoxins[1, 2]. There is relatively less information in the literature as to the chemical composition of toxoids; it is known that a preparation of toxoid of Bac. histolyticus is 70-100% protein [3].

The problem of the present work is to isolate and study chemically the toxoid of septic vibrio.

METHODS

Isolation of purified toxoid. The septic vibrio toxoid was obtained by detoxification of native toxin separated from the microbe body in the presence of 0.4% formalin for two days at 37°. The native toxoid was concentrated by precipitation with HCl in the isoelectric zone, with addition of NaCl [4].

After the precipitate had been washed three times with a 0.01 M acetate buffer, pH 5.0-5.1, it was dissolved in an 0.05 M acetate buffer, pH 5.4-5.8, with a volume 1/50 of the original. The native toxoid contained 0.1-0.5 units of activity per mg of nitrogen (the activity was determined in combining units, CU, with a standard specific antiserum), the concentrate contained 50-100 CU.

The concentrates obtained by selective solution in an acetate buffer were purified by repeated reprecipitation with acetone in the cold [5]. Then the precipitate was dissolved in 0.05 M Na acetate, concentrating it 4-5 times. The purified, concentrated solution of toxoid had a light yellow color. However, this preparation could not be considered a pure, homogeneous toxoid, since it could be divided by nitrogen content in the organic substances (from 20 to 80 mg%). It contained 200-400 CU per mg of nitrogen. The lyophilically dried, purified toxoid was fractionated with acetone which permitted separation from the heterogeneous materials of the purified concentrates of a number of preparations of toxoid with identical compositions and high activity.

Fractionation by acetone. The dry toxoid preparation was dissolved in distilled water (10 mg/ml) and precipitated by addition of increasing amounts of acetone. Each portion of acetone was added to the clear centrifugate after separation of the precipitate obtained from the preceding portion of acetone. The precipitate was dissolved in distilled water, once more reprecipitated by acetone, again dissolved in water, and dried by lyophilization. All these procedures were carried out at temperatures of 10-15°. The toxoid fractions were obtained at acetone contents of 40, 60, 80, and 100% (by volume). The first fraction was obtained by precipitation with 40% acetone and was slightly active. The fractions obtained using 60, 80, and 100% acetone had different activities and were studied chemically.

RESULTS

Chemical Composition and Some Physical Properties of the Toxoid

In the toxoid fractions obtained by precipitating with 60, 80, and 100% acetone with different activities, we determined the content of N_{total} with micro-Kjeldahl, and reducing sugars by the method of Hagedorn-Jensen. The results, given in the table, show that all the fractions contained similar amounts of nitrogen, but differed in activity, amount of reducing substances, and ash. Elementary analysis of the ash showed that its main component was iron.

The toxoid preparations were hydrolyzed (6 hours on on a water bath at 100° with 1 N H_2SO_4); after paper chromatography of the hydrolyzate, using as solvent butanol-acetic acid-water (40 : 10 : 50), we found in it mannose, glucose, and a trace of galactose. Determination of carbohydrates according to Hultman [6] permitted us to avoid the errors introduced by the presence of iron.

To 0.5 ml of hydrolyzate we added 0.5 ml of 40% solution of Na p-aminosalicylate and 5 ml of glacial acetic acid, stirred, and placed in a water bath for 20 min. Then the tube was taken out, cooled to room temperature and measured colorimetrically in a photoelectric colorimeter with a green light-filter. The concentration of sugars was determined from a standard curve. The method permitted determination of 0.05

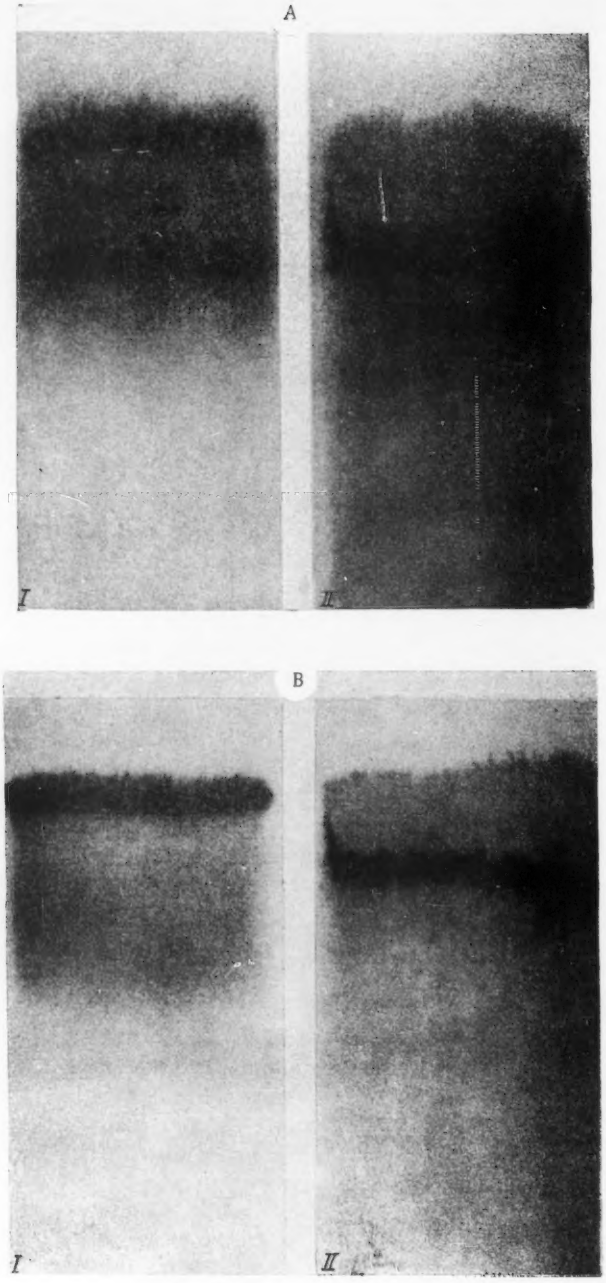

Fig. 1. Electrophoretogram of septic vibrio toxoid. A) Preparation 2, fraction 2; B) preparation 1, fraction 1. I) pH of buffer 6.8; II) pH of buffer, 5.3.

Composition of Septic Vibrio Toxoid

Preparation No.	Acetone conc. for ppt., %	Ash, % of absolute dry weight	In 1 mg of organic substance		
			N_{total}, mg	reducing substances as mg glucose	CU per mg N_{total}
I	60	7	0,084	0,126	600
	80	23	0,099	0,098	2800
	100	35	0,089	0,068	650
II	60	12	0,084	0,140	1200
	80	32	0,083	0,200	3000
	100	42	0,082	0,210	1150
III	60	37	0,075	0,150	750
IV	60	12	0,082	0,256	3000
	100	67	0,064	0,380	2500

mg/ml of glucose, galactose, and mannose. The results showed that in quantitative content of carbohydrates (in mg/g of organic substance), the difference between the separate preparations was slight:

Preparation No.	II		IV	
Fraction	2	3	1	2
Amount of sugar, mg	0,074	0,057	0,069	0,079

These results show that fractionation by acetone permits separation of toxoid into fractions with the same content of nitrogen and carbohydrates, but with different activities and different amounts of ash whose chief component is iron. No sulfur or phosphorus were found in the toxoid.

These toxoid preparations gave a positive biuret reaction with maximum absorption at 540 mμ; they were not precipitated by 5% TCA, but were precipitated by 10% TCA.

In paper chromatography of the amino acids of the toxoid hydrolyzate (in 6 N HCl, 36 hours on a sand bath) we found twelve spots which corresponded to the amino acids: lysine, arginine, aspartic acid (much), serine (much), hydroxyproline, glutamic acid (much), alanine, proline, tyrosine-aminobutyric acid, methionine-valine, phenylalanine, leucine.

A study of the ultraviolet spectrum of the toxoid showed differing peak heights at 275 mμ and their disappearance in preparations which were kept in solution and lost their activity. This fact indicates that the aromatic amino acids take part in the biological activity of septic vibrio toxoid.

In paper electrophoresis the isolated toxoid separated into two components. After six hours of electrophoresis in 0.1 M acetate buffer, pH 6.8 and 20 v/cm, from the main part of the toxoid which remained at the point of deposition there separated a fraction which was present in small amount in the toxoid (Fig. 1, A, I). In electrophoresis carried out under the same conditions but at pH 5.3, the fractions changed places (under conditions of depositing the sample in the center of the paper strip), Fig. 1, A, II. In some preparations the second (smaller) fraction did not appear (Fig. 1, B).

Immunological Analysis of the Preparations

We studied the antigen composition of the preparations in agar by the method of Ouchterlony [7]. As the antibody we used horse antiserum.

Bjorklund and Berengo [8] found in septic vibrio toxin 6-7 lines of precipitation. For the toxoid we found a different number of precipitation lines. Antigens of the same activity (70-100 CU/ml) gave with serum which contained 200 AU/ml and simultaneous introduction, six precipitation bands (Fig. 2b) and with serum containing 20 AU/ml two precipitation bands (Fig. 2a). Using equivalent concentrations and volumes of toxoid and serum in all preparations gave only one precipitation line, which did not separate in the three months of observation

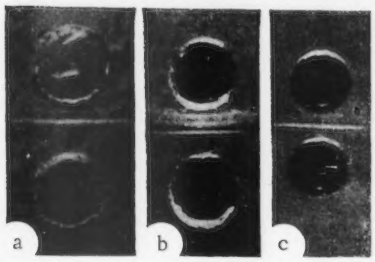

Fig. 2. Precipitation in agar of septic vibrio toxoid with antiserum. a) Precipitation of antigen with activity 100 CU/ml with antiserum 20 AU/ml; b) with serum with 200 AU/ml; c) with serum with 100 AU/ml.

(Fig. 2c). One precipitation line was always obtained when the reagents were used in equivalent concentrations and volumes, independent of the activity (preparations were studied with activities from 20 to 200 CU/ml).

SUMMARY

By concentration and purification of native septic vibrio toxoid by precipitation with HCl and acetone followed by fractionation with acetone we have obtained toxoid preparations with constant nitrogen-carbohydrate content. The total nitrogen is 8.2%, the carbohydrate 6-8% of the organic substance. Iron enters the composition of the toxoid preparations.

The protein component of the toxoid is a small molecule, approximating in size a polypeptide, and contains the following amino acids: lysine, arginine, aspartic acid, serine, hydroxyproline, glutamic acid, alanine, proline, tyrosine-aminobutyric acid, methionine-valine, phenylalanine, and two leucines. The toxoid protein is firmly bound to the carbohydrate component, in the composition of which occur mannose, glucose, and perhaps galactose.

The purified toxoid preparation has an absorption maximum in ultraviolet light at 275 mμ; loss of activity of the toxoid when its solutions are kept is accompanied by disappearance of the peak at 275 mμ. Paper electrophoresis of strips of toxoid results in separation into 1-2 components. Contact diffusion in agar of the purified antigen against specific antiserum shows from one to six bands of precipitation depending on ratio of concentrations of antigen and antibody used.

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OXIDATIVE PHOSPHORYLATION IN THE BRAIN EXPOSED TO OXYGEN UNDER INCREASED PRESSURE

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The universal toxic action of excess oxygen pressure on living organisms, occupying different positions in evolutionary development (microorganisms, plants, crustacea, insects, amphibia, reptiles, birds, mammals, etc), is a solidly established fact. Of all the animal tissues studied (rabbits, rats, guinea pigs) nervous tissue is the most sensitive to the action of high oxygen pressures, especially the cerebral cortex and cerebellum.

Our previous investigation of phosphate metabolism showed that animals (rats, guinea pigs) remaining in an atmosphere of high oxygen pressure undergo a 40-45% decrease in the adenosine triphosphate (ATP) content of the liver, heart, and kidneys, but the ATP level in brain remains unchanged. There is an insignificant decrease in the creatine phosphate (CP) level in the brain. The results we obtained for brain we explained by a decrease of ATP synthesis or its utilization [1].

The rate of the phosphorylation processes is a well-known indication of the effectiveness of the oxidative processes-respiration and glycolysis. The amount of ATP and other high-energy compounds, the magnitude of the phosphorylation coefficient P/O, the P^{32} uptake rate in high-energy and other phosphoorganic compounds, etc., can be applied to characterize the oxidative processes quantitatively.

The physiological significance of the synthesis, of ATP, uridinetriphosphate, cytidinetriphosphate, and other polyphosphates is explained by the fact that their energy is used directly for the accomplishment of biological syntheses and to assure specific physiological functions (muscular contraction, nervous stimulation, etc.).

The normal progress, route, and rate of aerobic phosphorylation, are determined by many factors, among which are oxygen, glucose, vitamin E, biotin, etc., intake, and the content of inorganic phosphorus (P_i), adenosinediphosphoric acid (ADP), potassium and magnesium, diphosphopyridinedinucleotide (DPN), thyroxin, insulin, hypophyseal hormones, adrenal hormones, etc.

No changes in the ATP content in brain was observed in some forms of hypoxia; namely, in hypoxia caused by decreased oxygen concentration in inspired air [2], in hypoxia, occurring as a consequence of conversion of 40-60% of the hemoglobin to methemoglobin, with acute disturbance of carbohydrate metabolism. The rate of ATP renewal is not affected in this case. Only the combination of methemoglobin with injected caffeine brought about a significant decrease in the ATP level. The author assumes that preservation of the ATP level when oxidative processes are decreased is determined by a decrease in its utilization as a consequence of the development of inhibition [3]. Gordienko and Frenkel' [4] have observed that the ATP level is maintained in brain, and in some cases is increased, in animals receiving nitrochlorobenzene, carbon monoxide, etc. Rosengart has not observed changes in the ATP level of the brain in convulsions caused by corasole [5].

In this paper, the rate of respiration and oxidative phosphorylation of the rat brain cerebral cortex under the influence of 6-8 atm of pure oxygen has been studied.

EXPERIMENTAL METHODS AND DISCUSSION

The experiments were carried out in two variations. In the first, the animals were subjected to the action of oxygen at 6 atm pressure, in a special pressure chamber, which brought them to a severe "terminal" condition. Violent convulsions followed after a while, which are characteristic of oxygen intoxication. After removal from the pressure chamber the animals were decapitated, the brain quickly removed, as much of the cerebral cortex removed as possible and slices prepared. The ability of these slices to bind P_i and to accumulate ATP during incubation was determined. Brain slices from control animals were investigated concurrently.

In the second variation of the experiment, slices of the cerebral cortex of control animals were directly subjected to the action of oxygen. The slices were incubated in a pressure chamber under 8 atm pressure pure oxygen for one hour. Slices incubated in vessels filled with oxygen at atmospheric pressure served as controls. At the end of the incubation P_i and ATP were determined.

The first variation of the experiment had as its object to establish whether oxygen under pressure could

TABLE 1. The Effect of Increased Oxygen Pressure on the Respiration Rate of Cerebral Cortex Slices of Rat Brain (Gaseous medium, oxygen; T = 28.5°. Respiration in μ liters oxygen consumed/100 ml fresh tissue/hour)

Control	Pressure 6 atm O_2	Control	Pressure 6 atm O_2
68,31	75,19	80,88	81,36
84,48	91,68	81,44	78,28
97,68	78,24	83,08	101,08
91,80	105,84	110,64	—
83,52	81,60	72,96	—
83,11	104,40	102,48	—
87,12	71,28		

cause irreversible changes in systems involved with the binding of P_i in the animal brain. The second variation of the experiment was to establish the indirect effect of high oxygen pressures on phosphorylative processes in incubated cells.

The slices were incubated in 3 ml salt mixture and glucose, according to McIlwain's formula [6]. In the first variation of the experiment the slices were incubated in Warburg manometric flasks 25 min at T = 28.5°. The flasks were previously saturated for 5 min with oxygen. The oxygen uptake of the slices at T = 28.5° was lower than at T = 38°, which is the temperature usually used in respiration experiments. The lower temperature slowed ATP decomposition and thus is recommended for the investigation of phosphorylative processes [7]. At the end of the incubation the flasks were quickly set in an ice-water mixture, the slices were washed three times with an ice-cold solution of 0.9% NaCl, fixed with TCA, homogenized and extracted 30 min in the cold. P_i was determined in the TCA filtrates by precipitation with a magnesium mixture, ATP as phosphorus after 10 min hydrolysis of the mercuric precipitate. ATP phosphorus was determined by the method of Kuttner and coworkers [8]; $SnCl_2$ was used as a reducing agent in this method. This method is usually 5 times more sensitive than reduction with eikonogen or ascorbic acid. Results are presented in Tables 1 and 2.

As may be seen from Table 1, the oxygen uptake rate for brain slices of control and experimental animals is practically the same.

Earlier, Stadie [9] also could not find differences in oxygen consumption of brain slices between control animals and animals killed after a 30-min stay in a pressure chamber at pressures of 8 atm pure oxygen. In Van Goor's experiments [10] the brain respiration of animals which had been affected by 5 atm of oxygen, then studied at normal pressure, did not differ from the brain respiration of control animals. Stadie, Dickens, Van Goor, Gershenovich and Krichevskaya [9-12] discovered in in-vitro experiments a steady lowering in the respiration rate of tissue homogenates and brain slices, which increased as oxygen pressure increased, as exposure time lengthened and the tissue was more finely pulverized. Gershenovich and Krichevskaya also found a decrease in respiration rate in in vivo experiments [13]. In experiments on intact cells Khill and MacLeod [14] found a decrease in CO_2 given off when oxygen pressures were higher than 1 atm in rats, mice and young rabbits. Bean [15] showed a marked decrease in oxygen consumption in dogs with oxygen pressures of 5 atm. Van Goor, comparing these experiments in vivo and in vitro, considered that results obtained in vivo were not sufficient evidence for the absence of changes in the respiration process in oxygen intoxication. This belief he supported by material from his laboratory on the effect of narcosis on

TABLE 2. Effect of Increased Oxygen Pressure on P_i Content and ATP in Incubated Slices of the Cerebral Cortex of Rat Brain (Time of incubation, 25 min; T = 28.5°; salt mixture + glucose)

Control				Pressure 6 atm O_2			
unincubated slices		incubated slices		unincubated slices		incubated slices	
I	II	I	II	I	II	I	II
16,90	—	12,70	—	16,95	—	13,3	—
13,35	—	9,12	—	14,98	—	13,1	—
12,93	—	—	—	16,44	—	13,54	—
14,70	—	12,90	—	17,60	4,04	12,83	—
16,20	4,03	12,96	4,33	19,83	2,17	14,90	3,30
17,58	2,69	13,00	3,44	17,92	1,80	12,04	2,97
16,52	3,38	12,79	3,72	17,12	—	13,28	3,54
19,40	3,74	14,21	4,30	16,10	3,47	11,77	2,71
17,84	0,97	12,07	1,29	16,48	1,41	12,81	1,05
16,19	2,13	13,92	2,70	19,00	1,87	—	—
18,72	1,70	16,83	2,53	—	2,36	11,60	3,08
13,50	—	9,20	—	14,07	1,32	—	2,18
20,80	—	17,30	—	22,90	3,27	11,25	3,51
17,73	1,5	8,36	2,20	19,14	2,48	14,32	4,70
14,45	2,30	—	2,30	15,37	4,44	11,00	5,82
14,25	1,90	7,85	1,90	—	—	—	—
—	2,57	13,90	4,12	—	—	—	—

Note. I, P_i; II, ATP; calculated in μg/100 mg tissue.

TABLE 3. The Effect of Increased Oxygen Pressure on the P^{32} and ATP Uptake Rate and P_i Fractions in Incubated Slices of Animal Brain (The average figures from 5 experiments are given)

	Control			Pressure 6 atm O_2		
fraction of P	activity, imp/min per 0.1 g	P, μg/100 mg	S.A. (specific activity)	activity, imp/min/100 mg	P, μg/100 mg	S.A. (specific activity)
P_i	1072	9,74	112	1049	9,62	108
ATP	271	2,63	105	312	2,92	112
Acid-soluble P	3232	—	—	3311	—	—

respiration in animals killed after deep narcosis. Their respiration rate in the brain did not differ from that of control animals, but there was no doubt of the metabolic changes on narcosis.

The data of Stadie, Van Goor, and our own is evidence of the absence of irreversible changes in respiratory enzymes activity from the effect of high oxygen pressures on the animals. The P_i content decreased by 22%, the ATP content increased 18% during incubation of the slices. Comparison of the results obtained for control and experimental animals did not show significant differences. The P_i in control animals decreased 23%, and in experimental animals 26%; ATP increased in control animals by 18%, in experimental animals 21.2%. It can be considered that with the low level of ATP (3-3.5 mg%), which was obtained for the incubated tissue, we could not have found changes in the ATP level because of expeimental errors.

In order to decide this question we carried out an analogous series of investigations using P^{32}. We started with the consideration that even with rapid breakdown of the ATP formed, the P^{32} uptake would correspond to the rate of synthesis in the quantities remaining.

The P^{32} in the form of $KH_2P^{32}O_4$ was introduced in a salt mixture in the amount of 1.77 μC in a flask. The P^{32} uptake rate was determined in acid soluble phosphorus, P_i and P_{ATP}. In the P^{32} experiments, the slices were washed after incubation in an ice-cold solution of 0.9% NaCl until the radioactivity disappeared in the wash liquid (13-15 times) and the solution was then fixed with TCA. In order to determine the P^{32} and P_i uptake rate, a carrier solution of KH_2PO_4 was introduced into the TCA filtrate by the addition of magnesium mixture. At 12-14 hours after precipitation the P_i precipitate was separated by centrifugation, washed many times, dissolved in 0.5 ml 0.1 N HCl and its radioactivity determined. To determine P^{32} uptake rate in ATP, the precipitate formed after mercuric acetate precipitation was washed many times, dissolved in 0.5 ml 0.1 N HCl and used for the determination of radioactivity. The radioactivity was recorded on a B apparatus with an end-window counter. The P^{32} uptake rate in the investigated fractions was calculated on a basis of 100 ml wet tissue. In addition the specific activity of P_i and ATP was calculated according to the equation:

$$\frac{\text{imp/min/ 100 mg tissue}}{\mu\text{g P/100 mg tissue}}$$

Results are presented in Table 3.

As can be seen from Table 3, the P^{32} uptake rate in all the fractions of phosphorus compounds studied is practically identical in experimental and control animals. Thus, results of experiments using P^{32} confirmed the absence of irreversible changes in the oxidative phosphorylation system from the effect of high oxygen pressures in animals. In hypoxia, caused by maintaining animals for long periods of time in a room with 7-8% oxygen, Smirnova and Chetverikov [16] did not find any variation in the P^{32} uptake rate in phosphorus compounds of the brain. Krebs and co-workers [17] after oxygen starvation of animals for two hours (hypoxia which corresponded to a height of 9,000-11,000 m above sea level) also did not find changes in P^{32} uptake rate in a number of phosphorylated compounds of the brain, even in those animals which were killed at the time of the experiments.

Thus, changes in the oxygen regime, hyperoxia, some forms of hypoxia, while causing very severe conditions in the animals, did not lead to irreversible changes in the phosphorylating system of the brain. It is interesting to note in this connection, that tissue hypoxia, caused by injection of xyanide, caused a significant decrease in the CP and ATP content in the brain [18]. Gershenovich and Krichevskaya have shown in in vitro experiments that respiratory systems in the brain resistant to cyanide are sensitive to the effect of high oxygen pressure. Combination of the effect of high oxygen pressures with multiple lethal doses of KCN in their experiments led to practically complete respiratory inhibition [12].

In as much as the synthesis of high-energy bonds during oxidation mainly occurs in stages requiring Fe-containing enzymes, then the resistance of these enzymes to hyperoxia, possibly, is the reason why the phosphorylating system of the brain is only slightly sensitive to the effect of high oxygen pressures. From this point of view, we were interested that the phosphorylation coefficient in the liver significantly decreases with oxygen intoxica-

tion [20]. In liver, in comparison with the brain, there is considerably more riboflavin, a more active catalase and much less cytochrome C [19]. The specific weight of cytochromic systems in the liver in the oxidation process is less and hence, the phosphorylating systems of the liver are more sensitive to the action of hyperoxia. On comparing the effects of high oxygen pressures (6 atm) on the phosphorylation processes in different organs, the special status of the brain is clearly shown:

Organs	ATP content, %	Oxidative phosphorylation rate, in vivo	Oxidative phosphorylation rate, in vitro
Brain	Unchanged	Unchanged	Increased
Heart	Decreased 45	" "	" "
Kidneys	Decreased 40.6	Small change	Not investigated
Liver	Decreased 45	Decreased by half	Increased

In the heart and kidneys of "hyperoxygenated" animals the ATP content decreased, but this decrease was probably caused by increased consumption of ATP, with maintenance of the ability of the tissue to synthesize it. In the liver, phosphorylative systems are more labile; correspondingly, ATP is decreased and there is an irreversible decrease in the chance of ATP synthesis.

Differences in the conditions of synthesis and utilization of high-energy phosphorus compounds in the brain and other tissues, which occur with high oxygen pressures in animals, are not observed when 6 atm O_2 is directly applied on tissue preparations.

In in vitro experiments with direct application of oxygen to incubating brain slices (Table 4), heart muscle homogenates and liver preparations, oxygen had a stimulating action on phosphorylation in all the investigated tissues.

The difference between results obtained in experiments in vivo and in vitro must be related to the participation of regulatory systems of the organism, (thyroid gland, hypophysis, adrenals, etc), in the development of the phenomenon of hyperoxia which indicates action on the phosphorylation processes.

TABLE 4. The Effect of Oxygen on P_i and ATP Content in Incubated Slices of Brain Cortex (in vitro)

Control		Pressure 8 atm O_2	
I	II	I	II
24,65	1,57	21,19	2,66
11,29	0,80	9,86	2,26
13,96	3,92	11,33	5,27
16,31	2,40	12,59	3,15
17,30	1,47	10,55	2,20
16,41	—	14,18	—
16,23	1,59	14,58	2,18
17,30	—	12,17	—
15,02	2,32	9,37	2,47

Note: I, P_i; II, ATP; calculated in μg/100 mg tissue.

It is also possible, that since in vitro in experiments the activity of ATP-ase of the brain decreases, then consequently this amount of ATP can be higher and the level of P_i lower, than in control slices.

The unique oxidative processes in the central nervous system, the significant compensatory possibilities, the probability of less subjection to hormone activity [21, 22] lead to maintenance of the phosphorylating systems of the brain.

SUMMARY

Respiration rate and oxidative phosphorylation of slices of cerebral cortex of the brain, taken from the rat, undergoing the action of 6 atm of pure oxygen, and slices from control animals were practically alike (the rate of the phosphorylation processes was based on inorganic phosphorus content, ATP, P^{32} uptake rate in inorganic phosphorus, ATP and acid-soluble phosphorus).

The oxidative phosphorylation rate of brain slices exposed directly to oxygen at 8 atm pressure increased 22% in comparison with control slices.

The effect of increased oxygen pressure on oxidative phosphorylation in different tissues was compared.

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THE EFFECT OF SOME LIPID FRACTIONS FROM THE THYMUS AND SPLEEN ON METABOLIC PROCESSES

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While the biochemistry of the thymus gland has been the subject of a number of investigations, much in this area has not been studied.

Parhon and co-workers [1, 2] have shown an inhibitory action of protein extracts of the thymus gland in experimental cancer.

According to our data [3], the injection of two fractions extracted from the thymus, the lipid and the total protein fractions, show an effect on the development of experimental cancer, the activity of the lipid fraction being higher. In one of our previous papers [4] we investigated the effect of experimental athymia and the action of the total protein extract of the thymus on phosphorus compounds of the blood and liver.

In the present paper we present results obtained by studying the action of some lipid fractions extracted from thymus, and the total lipid fraction extracted from spleen, on metabolic processes.

METHODS

Extraction of lipid fractions. The lipid fractions were extracted according to the method suggested by Thomas and co-workers [5] for the extraction of lipids from the brain, with some modifications. The various components were extracted from fresh calf thymus. By the above-mentioned method, we isolated lipid fractions of different chemical composition which were designated as Fractions A, B, C, D. Fraction A contained the predominantly neutral fats, glycerides, cerides, and in lesser amounts, sterols; Fraction B, predominantly sterols; Fraction C, phosphatides; and Fraction D, cerebrosides and sphingomyelins. The total lipid extract was obtained, from fresh pulverized spleen, by using such solvents as ethyl alcohol, ether, chloroform, and methyl alcohol; this extract we designated as "Extract S." The lipid fractions were dissolved in olive oil in such a manner that each milliliter contained 0.05 g lipids.

Experiments on animals. The experiments were carried out on half-grown rats weighing 200 g, which were separated into groups, according to the lipid fraction injected. Extracts A, B, C, and D were injected subcutaneously, at 1 ml per day for 24 days. Rats from the control group were injected with olive oil in the same quantities. After the animals were killed, the following determinations were carried out in the liver and the blood: oxygen consumption (QO_2), glycolysis, glycogen content, lactic acid, glucose, P^{32} uptake in total nucleoprotein phosphorus (P_{np}) and in lipid phosphorus (P_{lip}).

Oxygen consumption and glycolysis were determined according to Warburg's method, glycogen according to Good and Somogyi's method [6] and lactic acid according to the method of Barker and Summerson [7]. Animals designated for investigation with P^{32} were injected intraperitoneally with P^{32} in the form of a solution of NaH_2PO_4 to the amount of 30 microcuries /100 g body weight.

RESULTS

Study of the oxygen consumption (QO_2) of liver tissue after injection of lipid fractions into the animals (in μliters O_2/g fresh tissue) gave the following results:

Animals	No. of expts.	Lipid fractions			
		A	B	C	D
Control	100	0,039±0,001	0,033±0,003	0,042±0,001	0,037±0,001
Experimental	100	0,043±0,001	0,042±0,001	0,042±0,001	0,040±0,0008

The injection of various lipid fractions usually brought about insignificant changes in oxygen consumption in the liver and blood. However, it may be noted that there was an increase in oxygen consumption of 27% from the effect of liver Fraction C. Comparison of the rate of glycolysis (in μliters CO_2/g tissue) in the liver of intact animals and rats receiving lipid fractions, showed the following [next page]:

Animals	No. of expts.	Lipid fractions			
		A	B	C	S
Control	100	0,367±0,012	0,423±0,032	0,440±0,033	0,480±0,028
Experimental	100	1,119±0,010	1,263±0,125	0,278±0,063	1,834±0,123

These results indicate the effect of lipid fractions on glycolysis. Fractions A and B significantly increased glycolysis 205 and 198%, respectively, while Fraction C showed the opposite effect, decreasing glycolysis 37%. The total lipid extract, obtained from spleen, caused the greatest increase in glycolysis in the liver (282%).

Comparison of the data on liver glycogen content (in mg glucose per 100 g tissue) of the control and experimental animals showed the following results:

Animals	No. of expts.	Lipid fractions		
		A	B	C
Control	45	409,0±10,7	438,8±17,0	409,0±10,7
Experimental	45	410,8±78,8	234,7±19,4	612,4±92,3

Thus, injection of the lipid fractions caused significant changes in glycogen content, the direction of which depended on the injected fraction. For example, Fraction B caused a marked decrease in glycogen content (46%), but Fraction C a significant increase (49%). Injection of the lipid fractions extracted from the thymus did not cause significant changes in the lactic acid content in blood, with the exclusion of Fraction A, which increased lactic acid 49%. The contents of glucose in the blood (plasma and erythrocytes) is not affected by lipid fractions from the thymus and spleen.

The P^{32} uptake (in imp/min/g tissue) in P_{np} of the liver underwent significant changes, depending on the injected fraction: P_{np} radioactivity increased 52 and 37% respectively on injection of Fractions A and B and 119% under the influence of Fraction C:

Animals	No. of expts.	Lipid fractions			
		A	B	C	D
Control	40	464,2	498,8	362,8	421,6
Experimental	40	708,4	684,2	798	253

Injection of total spleen extract led to a decreased P_{np} radioactivity of 40%.

Changes in P^{32} uptake in phospholipids in blood were noted after injection of lipid extracts as expressed by an increase in the radioactivity (in imp/min/g tissue); particularly evident (250%) after injection of Fraction S:

Animals	No. of expts.	Lipid fractions			
		A	B	C	S
Control	40	4,80±0,21	4,82±0,22	4,80±0,21	4,80±0,20
Experimental	40	7,80±0,75	7,41±0,98	6,10±0,58	16,8 ±0,29

DISCUSSION OF RESULTS

Our results are evidence of the well-known effect of thymus and spleen preparations on the characteristics we studied; moreover they were varied and were dependent on the chemical nature of the preparations. The observed changes were noted both in liver and in blood.

Increase in O_2 consumption under the influences of liver Fraction B accompanied an increase in glycolysis in our experiments; this effect has already been remarked by Elliot and co-workers [8] in biochemical and histological investigations in vitro.

In respect to liver glycogen it is necessary to emphasize the direction of the changes in glycogen content and the rate of glycolysis in the liver under the influence of various injected preparations.

An increase in glycolysis was accompanied by decrease in glycogen content (Fraction B), and a decrease in glycolysis by an increase in glycogen content (Frac-

tion C). In contrast to the effect of the sterol fraction extracted from thymus on glycogen of the liver, Makarevich and co-workers [9] observed that octestrol and folliculin decreased glycogen content. Similar results were obtained by Parhon and co-workers [10]. From the work of Bomskev it may be concluded that the hormones extracted by him from the thymus decreased the glycogen content in the liver 50% [11]. However, Anselmino [12] and Rockenberger [13], attempting to reproduce Bomskev's experiments, did not always obtain identical results. The reason for these differences may be that thymus lipid extracts of thymus having a different composition were used. According to our data, fractions containing predominantly sterols decreased glycogen content in liver, but fractions containing predominantly phosphatides increased the glycogen content.

It is necessary to emphasize the parallelism existing between the action of Fractions A and B, and the more intensive action of Fraction C on the metabolism of liver phosphorus compounds. Thus, for example, a variation in P^{32} uptake in the form of P_{np} was observed which was evidence of the participation of thymus in phosphorus metabolism and confirmed our previous results [4]. In addition, the effect of thymus extracts on phosphorus metabolism depended on the chemical composition of the injected extract.

Our results show the significance of the well-known relationship between glycolysis and P^{32} uptake in nucleoproteins of the liver. As a result of the injection of A and C extracts, an increase in glycolysis and P^{32} uptake occurred. These results are evidence that the thymus is actively participating in phosphorus metabolism.

SUMMARY

Four fractions were separated from the total lipid thymus extracts which possessed different chemical compositions and had different effects on metabolic processes. The effect of these fractions was studied on O_2 consumption, on glycolysis, on glycogen content in the liver, nucleoprotein and lipid phosphorus. It was established that the total spleen lipid extract also affected the metabolic processes studied.

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METABOLISM OF THIAMINE-S^{35} PHOSPHORIC ESTERS IN ANIMAL ORGANISMS

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One of the important practical attainments of modern vitaminology is the study of the mechanism of action of vitamins as coenzymes in the animal organism and the use of some of them (cocarboxylases, in particular) for therapeutic purposes.

The coenzyme function of cocarboxylases (thiamine pyrophosphate-TPP) was discovered in 1911 by Neuberg and Karczag [1]. Subsequent numerous investigations of the catalytic action of TPP enabled Eakin [2] to discern this basic function of vitamin B_1 in the animal organism. It was further shown that TPP was necessary therapeutically in those cases where thiamine was not sufficiently effective as a consequence of injury to the processes of phosphorylation in the organism (in myasthenia [3], parenchymal hepatitis [4], multiple sclerosis [5], cardiac decompensation [6], and other diseases). The therapeutic preparation TPP was first obtained in Italy [7]. In our country, more attention has been paid to the problem of obtaining vitamin-coenzymes. The technology of TPP production, in particular, has been worked out. However, to obtain TPP even in the form of an incompletely pure preparation, is a difficult task, since TPP is hard to separate from other phosphate esters, forming on phosphorylation of thiamine. In addition, the yield of TPP in this process is less than 10% of the original thiamine [8]. The above circumstances have raised the price production many times and increased its cost, compared with thiamine.

In our investigations, we used the method of Viscontini and co-workers [9], for synthesizing thiamine phosphate esters, securing almost a quantitative yield in the form of a mixture of thiamine triphosphate (T-3-P), thiamine pyrophosphate, (TPP), thiamine monophosphate (TMP) and thiamine tetraphosphate (T-4-P). In this paper, we have decided to determine in what measure the metabolism of the thiamine phosphate esters from the mixture (T-3-P, TPP, and TMP) differs from the metabolism of thiamine, when they are injected parenterally.

The basis for such a series of investigations is the work of a number of authors, who found in various animal tissues, aside from TPP, some T-3-P [10-13] and TMP [10, 11, 13]. Thus, T-3-P and TMP appear to be natural metabolites of vitamin B_1 in the animal organism. An enzyme T-3-P (myosin-thiamine-triphosphatase) was discovered in skeletal muscles of the animals which actively cleaves to TPP and orthophosphate [12, 14, 15]. Coenzyme properties of pure T-3-P are not yet known [16]; however, it quickly acquires catalytic activity because of the ease of its enzymatic hydrolysis to TPP [17, 18]. TMP, which is formed by the action of thiamine pyrophosphatase on TPP in various organs and tissues of animals, is not a coenzyme for decarboxylase [16, 17], obviously, thiamine pyrophosphatase is necessary for activity of the cells. This is particularly evidenced by investigations [19-21] which found that there was a significant increase in thiamine pyrophosphatase activity and decrease of TPP in tissues of B_1-avitaminotic animals. TMP acquires a catalytic coenzyme function when oxidized to disulfide [22].

METHODS

In order to study the metabolism of thiamine and its esters, we used thiamine-S^{35} and pure S^{35}-T-3-P, S^{35}-TPP, and S^{35}-TMP synthesized from thiamine by our method [23]. The deposition and decomposition of thiamine-S^{35} were compared with S^{35}-T-3-P, S^{35}-TPP and S^{35}-TMP after subcutaneous injection of equivalent doses of the pure compounds in various animals (white mice, rats, and guinea pigs). In addition to this, the deposition and decomposition of thiamine-S^{35} and equivalent doses of its labeled phosphate esters injected as a mixture of unseparated components(T-3-P, 30.0%; TPP, 30.6% TMP, 27.8%, T-4-P, 6.9%; and small quantities of other polyphosphate esters of thiamine-S^{35}) were compared; they were obtained by us according to a modification of the method of Viscotini and co-workers. Thiamine-S^{35} and its phosphate esters were injected in equimolecular quantities subcutaneously in 0.8% NaCl at a physiological dose-0.5 μg/g animal weight. At 24 hours, when the excess of the injected vitamin is excreted in the urine [24], the distribution of the labeled compounds was investigated in the organism of animals and in the urine excreted by them during the experiment.

RESULTS

Comparison of the Metabolism of S^{35}-T-3-P, TPP,-TMP and Thiamine-S^{35} Deposition and distribution in the Organism of S^{35}-T-3-P, -TPP, -TMP and-thiamine.

In order to study this problem we limited the determination in animal tissues only to total sulfur radioactivity (S^{35}_{tot}), since the nonvitamine products of thiamine decomposition are quickly excreted in the urine [25-28], and the proportion of nonvitamine radioactive sulfur assimilated by tissue proteins from total S^{35} in the tissue amounts to 1% of the radioactivity [29]. The remaining S^{35} of the tissues consisted of the vitamin-active products of thiamine-S^{35} [30, 13].

The deposition of thiamine-S^{35} and labeled T-3-P, TPP, and TMP in the whole organism and in the internal organs was studied in experiments on white mice. A total of 276 white mice (males) weighing from 18-23 g, which were kept on a general ration in separate vegetating beakers, were used for these experiments. For the determination of S^{35}_{tot} in mouse carcasses, the mice were killed with a lethal dose of ether, washed 5 min in running water to remove the radioactive excrement from the coat; each animal was placed in a beaker with a mixture of reagents (10 ml Benedict's reagent [31], 20 ml of concentrated HNO_3 and 30 ml of concentrated HCl) to complete dissolution of the animal tissues (solution is speeded up by boiling the mixture). After cooling, the liquid was decanted into a graduated cylinder; the layer of fat was extracted with 10 ml of hot Benedict's reagent, after which the S^{35} residues were extracted with hot water. The cooled washed liquid was combined with sufficient solution to bring its volume to 50 ml. Two 1 ml samples were removed from this solution to porcelain crucibles and further processed according to Benedict's method for the determination of S^{35}_{tot} [31]. The radioactivity of $BaS^{35}O_4$ extracted by this method was recorded in a B apparatus with an end-window counter. In order to determine S^{35}_{tot} in the internal organs contained in the thoracic and peritoneal cavities (excluding the large intestine and the urinary bladder) and brain, homogenates were prepared of the combined organs with sufficient concentrated ammonia to make a volume of 50 ml. The homogenate stood for several days to allow complete solution of insoluble clumps of tissue, after which it was deposited on stainless steel planchetts in 0.5 ml quantities, dried at 90° and the total radioactivity determined under the end-window counter of a B apparatus. Statistically treated [32] results of the investigation are presented in Table 1.

Results of the investigations showed (Table 1) that thiamine-S^{35} phosphate esters were retained to a larger degree in the intact organism of mice and in their internal organs than was thiamine-S^{35}, injected in an equimolecular dose. These differences are completely reliable and significant ($t > 2$); thus, an increase in the deposition of thiamine phosphate esters is more evident in the internal organs than in intact mouse carcasses.

TABLE 1. Deposition of S^{35}_{tot} in the Carcasses and Internal Organs of White Mice 24 Hours after the Injection of T-3-P, TPP, and TMP in Doses Equivalent to 0.5 μg/g Thiamine-S^{35} (Deposition in % of injected dose)

Injected	Carcasses		Internal organs	
	M	t	M	t
S^{35}-T-3-P	48,7	22,4	33,4	23,2
S^{35}-TPP	46,1	10,5	32,7	18,3
S^{35}-TMP	39,6	5,7	26,9	9,8
S^{35}-thiamine	34,8		23,7	

Note. In this and succeeding tables the arithmetical mean and the index of reliability are reduced to the difference of two airthmetical means: $t = \frac{M_1 - M_0}{\sqrt{m_0^2 + m_1^2}}$; M_1 and m_1 are respectively the arithmetical means and the average error of the arithmetical means of the corresponding thiamine phosphates; M_0 and m_0 are the arithmetical mean and the mean error of the arithmetical mean for thiamine.

According to the amounts deposited in intact animals and in the internal organs of white mice, the phosphate esters are arranged in the following order: T-3-P > TPP > TMP > thiamine.

Distribution and deposition of thiamine-S^{35} and its labeled esters in separate organs was investigated in white rats and guinea pigs.

A total of 112 white rats, were used for these experiments (males of a single line with weights from 195-220 g, which were placed on a general ration, and on the day of the experiment received 50 ml of whole cow's milk) and 71 guinea pigs; (males of weights from 385-425 g, which were placed on a general ration, and on the day of the experiment received 60 g fresh carrots). At 24 hours after subcutaneous injection of equimolecular doses of labeled T-3-P, TPP, TMP, and thiamine, the animals were decapitated and weighed portions of 400-495 mg were obtained from liver, right kidney, small intestine (a section of jejunum, located 10 cm below the duodenum), the right hemisphere of the brain, the heart muscle in the region of the left ventricle, and skeletal muscle in the region of the right femur. In experiments in which thiamine-S^{35} having a low specific activity (less than 100 μC/g) was used, we concentrated the S^{35} of the organs and tissues; weighed portions were placed in test tubes; 2 ml Benedict's reagent, 4 ml concentrated HCl, and 6 ml concentrated HNO_3 were added to each tube to dissolve the tissue. The remaining tissue was treated with a mixture of the above indicated reagents until solution occurred. The contents were then quantitatively transferred to a porcelain watch glass, the test tube washed with another 5 ml of Benedict's reagent, and $BaS^{35}O_4$ precipitated from all the weighed portions

TABLE 2. Deposition of S^{35}-T-3-P, -TPP and -TMP in White Rat and Guinea Pig Organs 24 Hours after their Injection in Doses, Equivalent to 0.5 μ g/g Thiamine-S^{35}

Investigated organ	RA of organs and tissues after injection						
	S^{35}-T-3-P		S^{35}-TPP		S^{35}-TMP		thiamine-S^{35}
	M	*t*	*M*	*t*	*M*	*t*	*M*
White rats							
Liver	340	7,4	336	6,9	309	3,6	284
Kidneys	215	4,7	202	2,8	200	2,5	177
Small intestine	89	4,5	86	4,6	79	3,5	67
Brain	96	4,2	92	3,3	86	2,4	70
Heart muscle	197	4,5	177	3,4	169	2,2	145
Skeletal muscle	26,3	2,2	26,1	2,1	25,2	1,4	22,5
Guinea pigs							
Liver	218	2,9	213	2,7	191	1,6	173
Kidneys	264	2,4	266	2,1	239	1,2	218
Small intestine	120	2,6	115	2,0	106	0,8	101
Brain	89	3,3	86	2,8	72	0,7	68
Heart muscle	113	3,2	112	3,1	92	0,8	87
Skeletal muscle	27,7	2,0	27,4	2,0	23,2	0,5	22,1

according to Benedict's method. When thiamine-S^{35}, with a specific activity from 100 to 1000 μ C/g was used in the experiments, homogenates were prepared from weighed portions of the organs in concentrated ammonia, the volume of which was made up to 5.0 ml. The homogenate remained in ammonia for 24 hours to allow complete solution of the insoluble clumps of tissue (to calculate radioactivity; a 0.5 ml sample of homogenate was taken). As a primary criterion of the deposition of S^{35}-T-3-P, S^{35}-TPP, S^{35}-TMP, and thiamine-S^{35}, we selected the relative specific activity which was abbreviated to relative activity (RA).

$$RA = \frac{\text{imp/min in 1 g tissue} \times 100}{\text{imp/min, injected per 1 g of animal weight}}$$

The statistically treated results of these investigations are presented in Table 2.

At 24 hours after injection, the distribution of thiamine-S^{35} in the internal organs and tissues of the white rats and guinea pigs was entirely unequal. Thiamine-S^{35} in white rats was very heavily deposited in liver, kidneys, and heart muscle, moderately in brain and small intestine, and very lightly in skeletal muscle. Organs and tissues of white rats were arranged in the following order, according to the rate of thiamine-S^{35} uptake: liver > kidneys > heart muscle > brain > small intestine > skeletal muscle. Similar inequality of distribution in organs and tissues of white rats was observed at 24 hours after injection of equimolecular quantities of T-3-P, TPP, and TMP, the only difference being that thiamine phosphate esters in almost all investigated organs were deposited more heavily than thiamine. These differences were inconsiderable, but were completely significant and reliable for the majority of investigated organs and tissues of white rats. These differences were especially distinct in liver, somewhat less distinct in the remaining organs, and very slight in skeletal muscles, where statistical reliability of the difference was observed only when the deposition of thiamine was compared with T-3-P and TPP.

According to the amount deposited in rat internal organs, vitamine B_1 forms are arranged in the following order: T-3-P > TPP > TMP > thiamine. A similar increased deposit of T-3-P, TPP, and TMP in comparison with thiamine were observed in guinea-pig organs. It must be noted that the distribution of thiamine-S^{35}, and its phosphate esters also, was somethat lower in guinea pig organs than in white rat organs. The guinea pig organs are arranged in the following order, according to the amount of deposition of thiamine S^{35} or its phosphate esters: kidneys > liver > small intestine > heart muscle > brain > skeletal muscle. A statistically reliable and significant increase in deposition was observed when injected T-3-P and TPP were compared with thiamine. A larger deposit of T-3-P and TPP than thiamine was especially marked in brain and heart muscle, well-defined in the liver, and only slightly apparent in kidney tissue, small intestine, and skeletal muscle. Deposition of TMP in guinea pig organs did not differ significantly from thiamine deposition.

Decomposition of S^{35}-T-3-P, -TPP, -TMP, and Thiamine in the Animal and Urinary Excretion of Labeled Metabolites In investigations [25-28] carried out with the injection of labeled thiamine into humans, dogs, rabbits, guinea pigs, and white rats, it has been shown that numerous radioactive products of its metabolism are excreted in the urine. Among the thiamine metabolites in urine the following have been identified: Unchanged thiamine, thiamine disulfide, 4-methyl-5-beta-hydroxyethyl thiazole, thiochrome, and sulfates. The possible urinary excretion of thiamine phosphate esters has been disputed in a number of papers [30].

TABLE 3. Excretion of S^{35} in the Urine for 24 Hours after Injection of T-3-P TPP, and TMP in Doses Equivalent to 0.5 μg/g Thiamine-S^{35}. (In % excretion of the injected dose)

Fraction-S^{35}	S^{35}-T-3-P		S^{35}-TPP		S^{35}-TMP		Thiamine
	M	*t*	*M*	*t*	*M*	*t*	*M*
White rats							
Total	39,2	4,0	37,3	4,8	41,0	2,7	44,8
Thiamine	18,8	2,6	18,0	2,7	15,9	1,0	14,7
Sulfates	0,80	5,4	0,83	5,3	1,17	3,4	2,06
Guinea pigs							
Total	32,1	4,0	30,9	5,0	34,7	3,1	41,6
Thiamine	13,2	3,2	14,0	3,2	13,4	2,3	10,4
Sulfates	0,93	10,7	0,93	10,7	1,24	8,0	2,44

The circumstance that thiamine and its decomposition products are excreted in the urine, the most predominant of these products being 4-methyl-5-beta-hydroxyethyl thiazole [26, 28], permits urinary analysis to serve as a basis of investigation of the decomposition rate of T-3-P, TPP, TMP, and thiamine in the animal organism.

The urine of white rats and guinea pigs (the same animals in which the internal organs were investigated) was collected for 24 hours following the injection of equivalent doses of labeled T-3-P, TPP, TMP, and thiamine. The animals were placed in a large Buchner funnel for urine collection. The urine was stabilized by the addition of 2 ml 0.1 N HCl and the following determinations were made: 1) S^{35}_{tot} after evaporation of 0.1-0.5 ml of urine with 1-2 drops of concentrated ammonia on planchettes at 90°; 2) sulfate-S^{35}, precipitated from urine in the form of $BaS^{35}O_4$, in amounts less than 10 mg (in order to avoid erros because of S^{35} beta-ray absorption); 3) thiamine-S^{35}, extracted from urine with isobutyl alcohol in the form of thiochrome-S^{35} according to Eliseeva's method [33]. Part of the alcohol extract, containing thiochrome-S^{35} was evaporated on steel planchettes and the radioactivity measured according to our method.

Comparing the S^{35}_{tot} in the urine with the radioactivity of thiamine-S^{35}, the urinary excretion of thiamine-S^{35} metabolites could be calculated:

$$S^{35}_{tot} - \text{thiamine-}S^{35} = \text{thiamine-}S^{35}\text{ metabolites.}$$

Thus, by comparison of S^{35}_{tot} with the radioactivity of thiamine-S^{35} in the urine, it is possible to determine its decomposition rate into other, basically nonvitamin, products. In addition to this, we determined radioactive sulfates in urine, which can be considered as one of the final products of thiamine-S^{35} decomposition. The oxidation rate of the reduced thiamine sulfur to sulfates is also evidence of the degree of its destruction in the animal organism [27, 29].

Reasoning from the above statements brought us to the conclusion that the determination of S^{35}_{tot}, thiamine-S^{35}, and sulfate-S^{35} in the urine made it possible to study the decomposition rate of T-3-P, TPP, TMP, and thiamine labeled with S^{35} and injected into the animal. The statistically treated results from the urine of animals receiving T-3-P, TPP, TMP, and thiamine are presented in Table 3.

Thiamine-S^{35} is excreted from white rats at 24 hours in larger quantities (44.8%) than TMP (41.0%) TPP (37.3%) and T-3-P (39.2%), injected in equimolecular doses. These differences are quite small, however, they are statistically reliable and confirm earlier data showing a larger deposition of T-3-P, TPP, and TMP in animal tissues compared to thiamine. Similar results were found by determination of S^{35}_{tot} excretion in guinea pig 24-hour urine, the only difference being that guinea pigs deposit thiamine and its phosphate esters (T-3-P, TPP, TMP), in somewhat larger amounts than do white rats.

Results of the investigation showed that urinary excretion of thiamine-S^{35} in white rats and guinea pigs injected with T-3-P, TPP, and TMP is higher in comparison with animals receiving thiamine (animals receiving TMP had less urinary thiamine-S^{35} than those receiving T-3-P and TPP). This is not explained by a more active excretion of vitamin B_1 from the organism (S^{35}_{tot} excretion in animals receiving T-3-P, TPP, and TMP was less than after injection of thiamine-S^{35}), but by retardation of the decomposition of these components into nonvitamin products in the animal organism. This relationship is particularly apparent if the urinary ratios are calculated in terms of percent of thiamine-S^{35} and S^{35}_{tot}; S^{35}_{tot} being taken as 100%. The calculation showed that in white-rat urine, collected for 24 hours after injection of thiamine-S^{35}, the ratio of thiamine-S^{35} to its metabolites is 32.8: 67.2%, after injection of TMP, it is 38.7 : 61.3%, after injection of TPP, it is 48.2 : 51.8% and after injection of T-3-P it is 47.9 : 52.1%.

These data convincingly demonstrate that TPP, T-3-P, and TMP are more-slowly broken down in white rats into nonvitamin products than is thiamine. Identical data were obtained in experiments on guinea pigs, in

which the destruction of thiamine and its phosphate esters occurred more rapidly. The ratio of thiamin-S^{35} and its metabolites in guinea pig urine on injection of thiamine as 25.0 : 75.0%, on injection of TMP, 38.6 : 61.4%, on injection of TPP, 45.3 : 54.7%, and on injection of T-3-P, 41.1 : 58.9%.

Differences in the metabolism of T-3-P, TPP, TMP, and thiamine were especially significant when their decomposition into sulfates was investigated. Both in white rats and guinea pigs (in which thiamine-S^{35} and its phosphate esters are more actively oxidized to sulfates) considerably larger destruction of the parenterally injected thiamine was found than TMP, or especially, TPP and T-3-P.

These differences are seen when the percentage excretion of sulfate-S^{35} from injected doses of labeled thiamine and its phosphate esters (Table 3) are compared. The percent excretion of radioactive sulfates in 24-hour urine from injected doses was much higher after injection of thiamine (2.06%).than after injection of TMP (1.17%), TPP (0.83%), or T-3-P (0.80%) in white rats, and correspondingly after injection of thiamine (2.44%), TMP (1.24%), TPP (0.90%), and T-3-P (0.83%) in guinea pigs. Decomposition of thiamine-S^{35} and its phosphate esters is still more demonstratively characterized by comparison of the urinary excretion of thiamine-S^{35} and sulfate-S^{35}. From this it is clearly seen how much of the unchanged vitamin is excreted in the urine and what part of it is decomposed to sulfate. Calculations showed that in white rat urine, collected for 24 hours after injection of thiamine-S^{35}, the ratio of thiamine-S^{35} to sulfate-S^{35} is 7.1 : 1, after injection of TMP, 13.7 : 1; after injection of TPP, 21.1 : 1; after injection of T-3-P, 23.5 : 1. In guinea pigs, respectively; 4.2 : 1 after injection of thiamine-S^{35}; 10.8 : 1 after injection of TMP; 15.0-1 after injection of TPP; and 14.3 : 1 after injection of T-3-P.

The urinary excretion of S^{35}_{tot}, thiamine-S^{35} and sulfate-S^{35} was also investigated as well as fecal excretions of S^{35}_{tot} at 6, 24, 48, and 72 hours after white rats were injected with T-3-P, TPP, TMP, and thiamine-S^{35}. The data obtained in these investigations confirmed the conclusion drawn above that the deposition of T-3-P, TPP, and TMP was higher, and their decomposition less in comparison with thiamine.

Comparison of the Metabolism of Thiamine-S^{35}, S^{35}-TMP, -TPP, -T-3-P, -T-4-P and S^{35}-Thiamine Polyphosphates, Injected in a Combined Form.

When T-3-P is obtained by the method of Viscotini and co-workers [9] a pure preparation is not formed, but instead a mixture of different thiamine phosphate esters; among these, T-3-P is the most predominant. There is a considerable amount of TPP, less TMP, T-4-P and traces of other thiamine polyphosphate esters. In our previous work [23] the complicated composition of T-3-P obtained by this method was hypothesized and electrophoretic investigation of labeled products of its synthesis presented. It must be noted, however, that using a modification of Viscotini's method applicable to small initial quantities of thiamine-S^{35} (5-40 mg), we did not succeed in maintaining the volumetric and quantitative relationship of the reagents propounded by the authors of the method. Thus, in our mixture of thiamine phosphate esters a somewhat lower yeild of synthetic components was observed: T-3-P, 30.0%; TPP, 30.6%, TMP, 27.8%, T-4-P, 6.9% and the remaining 4.7% consisted of thiamine penta- or -hexaphosphate, unidentified thiamine polyphosphate esters; and also a residue of unreacted thiamine-S^{35} [23].

Because of our discovery that T-3-P, TPP, and TMP are deposited to a larger extent and decomposed less by the animal organism in comparison with thiamine, and taking into account that pure preparations of T-3-P, TPP, and TMP were very difficult to obtain in practice, we decided to study the possibility of using a mixture of thiamine phosphate esters in therapeutic practice since this mixture can be obtained in almost quantitative yield from thiamine-S^{35} by Viscontini's method. In order to test this possibility we investigated the metabolism of thiamine-S^{35} phosphate esters obtained by Viscontini's method in the form of a mixture containing, as described above, TMP, TPP, T-3-P, T-4-P, and thiamine-S^{35} polyphosphate esters (the experiments were carried out on 154 white mice and 39 white rats).

A study of the S^{35}_{tot} deposition in carcasses and internal organs of white mice 24 hours after the injection of a mixture of thiamine-S^{35} phosphate esters in a dose equimolecular with 0.5 $\mu g/g$ of thiamine-S^{35}, gave the following results (deposition in % of the injected dose):

Injected	Deposited in carcasses		Deposited in internal organs	
	M	*t*	*M*	*t*
Thiamine-S^{35}	39,2	—	22,9	—
Mixture of thiamine-S^{35} phosphate esters	44,0	5,4	26,5	5,7

The mixed thiamine phosphate esters were deposited to a larger extent in the internal organs (Table 4) and less was decomposed (Table 5), than thiamine-S^{35}. These differences were less distinct than after injection of pure T-3-P and TPP; however, they were completely significant and reliable. An increase in deposition of the mixed thiamine-S^{35} phosphate esters did not differ significantly from the deposition of thiamine-S^{35} only in the liver and kidneys of white rats. It must be taken into account that the mixed labeled thiamine-S^{35} phosphate esters which we studied contained less T-3-P and significantly more TMP than preparations obtained in large quantities by Viscontini's method. Thus, one must anticipate that preparations of a mixture of thiamine phosphate esters, in which T-3-P and TPP clearly predominate, will have a larger deposition and less de-

TABLE 4. Deposition in Organs of White Rats of Thiamin-S^{35} Phosphate Esters 24 Hours after Injection in the Form of a Mixture in Doses Equivalent to 0.5 μg/ /g Thiamine-S^{35}

Organs and tissues	RA after injection		
	mixture of thiamine-S^{35} phos. esters		thiamine S^{35}
	M	t	M
Liver	293	1,8	271
Kidneys	201	1,6	188
Small intestine	74	6,1	63
Brain	81	5,5	70
Heart muscle	163	3,3	148
Skeletal muscle	20,9	2,7	20,5

composition in the animal's organism than the mixture we studied.

DISCUSSION

The parenterally injected thiamin phosphate esters (T-3-P and TPP especially) are retained in larger measures in animal tissues, and a lesser amount of thiamine, injected in an equivalent dose, is excreted in the urine. Such retarded excretion of T-3-P, TPP, and to a lesser degree, TMP can be explained by their lower penetration [2] of the cellular membrane, and, in particular, the renal membrane. This factor has a great significance, since the main loss of parenterally injected vitamin B_1 is related to its urinary excretion in the first hours after injection when its concentration in the blood is markedly increased. On the other hand, TPP and T-3-P (which are quickly hydrolyzed to TPP in animal tissues) represent coenzymes, bound to apoenzymes and, thus, relatively more able to be retained in animal tissues than TMP and thiamine.

Thiamine phosphate esters (T-3-P and TPP particularly) are more slowly decomposed into nonvitamin products than thiamine. We propose that in animal tissues thiamine phosphate esters in general are not decomposed into nonvitamin products (or are decomposed in very small quantities), and only after their hydrolysis to thiamine begin to decompose to nonvitamin products. This is confirmed by the fact that urinary excretion of thiamine-S^{35} occurs after injection of S-labeled T-3-P, TPP, and TMP into animals. According to our hypothesis, metabolism of parenterally injected thiamine or thiamine phosphate esters also differ in that thiamine begins to decompose into nonvitamin products immediately after injection, and phosphate esters only decompose after a certain time necessary for their preliminary hydrolysis to thiamine. As a result, thiamine phosphate esters and thiamine can be arranged in the order T-3-P < TPP < TMP < thiamine according to rate of decomposition into nonvitamin products, which

TABLE 5. Excretion of Radioactive-Sulfur in Urine after Injection of Thiamine-S^{35} Phosphate Esters into White Rats in the Form of a Mixture in Doses Equivalent to 0.5 μg/g Thiamine-S^{35} (% of excretion for 24 hours of the injected dose)

Fraction S^{35}	Mixture of thiamine-S^{35} phosphate esters		Thiamine-S^{35}
	M	t	M
Total	42,6	2,5	47,4
Thiamine	24,1	5,2	17,9
Sulfates	0,78	9,2	1,40

is confirmed by our experiments on white rats and guinea pigs.

The most valuable results of our data can be considered the demonstration that TTP is deposited to a larger extent and more slowly decomposed in the animal organism to nonvitamin products in comparison with TPP and especially with TMP and thiamine. This fact can have great practical significance, if the parenteral injection of the phosphate esters, and particularly, the triphosphate ester, T-3-P, is recommended in therapeutic practice, as well as the injection of thiamine. In addition to the positive qualities enumerated above, T-3-P, similar to adenosine triphosphak (ATP), is a high energy compound, capable of accumulating energy from carbohydrate oxidation (and, in particular, pyruvic acid) and exchanging with gamma- and, to a lesser extent, beta-phosphate high energy with other high energy compounds. It is especially important to emphasize that chemical synthesis and industrial production of T-3-P are considerably simpler and cheaper than the production of TPP (cocarboxylase). The presence of small quantities of TMP and thiamine polyphosphate esters in preparations of T-3-P and even TPP does not decrease their therapeutic properties, since TPP possesses considerable biological activity as a coenzyme; TMP is more easily converted into TPP in animal tissues, than thiamine; a small quantity of T-4-P and other polyphosphate esters of thiamine are quickly hydrolyzed to T-3-P and then to TPP in the animal organism.

SUMMARY

Parenteral injection of physiological doses of thiamine triphosphate (T-3-P), thiamine pyrophosphate (TPP) and in less degree thiamine monophosphate (TMP) are retained better in organs and tissues of the animal organism (white mice and rats, guinea pigs), than thiamine injected in equimolecular doses.

T-3-P, TPP, and TMP are more slowly decomposed in the animal organism into nonvitamin products than thiamine.

Thiamine injected parenterally for therapeutic purposes can be replaced successfully by T-3-P or TPP,

which will be more effective, since they are better retained by the animal tissues and decomposed less into nonvitamin products.

The use of T-3-P for parenteral injection for therapeutic purposes is of practical importance since its chemical synthesis and industrial production are simpler and its cost significantly less (almost a quantitative yield is obtained) than TPP (cocarboxylase); preparations containing other thiamine phosphate esters also possess higher biological activity than thiamine.

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THE ISOLATION OF RIBONUCLEIC ACID FROM THE PLASMA SAP OF PLANT LEAVES AND A STUDY OF THE PECULIARITIES OF ITS METABOLISM

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The particular low-molecular weight, soluble RNA found in the supernatant fraction of cell homogenates [1-5] has attracted much attention from biochemists. In a comparatively short time (1958-1959) and as the result of intensive work in a number of laboratories [6-10] the main chemical properties and biochemical function of this RNA have been established.

It was shown that the soluble RNA fraction localized in the hyaloplasm of practically all animal, plant, and bacterial cells contained about 10-20% of all the RNA of the cell cytoplasm. In distinction from the high polymer RNA isolated from cellular organoids (molecular weight from 200,000 to 1,500,000), the fraction of soluble RNA is a comparatively small polynucleotide constructed from 40-80 nucleotide units. In spite of biochemical and chemical heterogeneity, the molecules of soluble RNA have a general characterstic: each of them has the same terminal trinucleotide grouping, constructed by a specific enzyme and consisting of two cytidylic and a terminal adenylic nucleotide [11].

It has been established that molecules of soluble RNA, reacting with activating enzymes, accept the aminoacyadenylates found on their surface. The amino acid residue of the aminoacyladenylate is united to the 2 or 3 carbon atoms of the ribose end in the adenyl nucleotide of the soluble RNA, and the adenyl component of the aminoacyladenylate is set free in the cell solution.

After accepting the activated amino acids, the molecule of soluble RNA then reacts with the high polymer RNA in the organoids, which fulfills the function of a stable matrix, and this reaction assures the uptake of proteins in protein synthesis. The latter stages of this process are still insufficiently studied, but, from a number of theoretical suggestions, it is the character of the reaction between the two forms of RNA (soluble and organoid) which determines the distribution of the amino acids in the polypeptide chains [6, 12-14].

The nature of these intermediate reactions which occur with the participation of soluble RNA and have been considered in more detail in a number of reviews [12, 15-17] clearly show the great value of a study of these processes for complete discovery of the mechanism of protein synthesis and the role of the different forms of RNA in reproducing their specificity. However, the study of soluble RNA is connected with a number of methodological difficulties which limit the possibility of experimental work in this direction in many biochemical laboratories which do not have rapid refrigerated supercentrifuges. In order to obtain the supernatant fraction of homogenate, more or less free not only from microsomes, but from the polymeric RNA of microsomes which has partly dissolved in the homogenization, it is necessary to centrifuge the homogenate with cooling at 105,000 × g for 2-3 hours.

In work with plant materials we have attempted to avoid this complex procedure and to simplify and hasten somewhat the method of isolating the fraction of soluble RNA in a large amount of so called plasma sap of plants, obtained by the method of Mason and Phillips [18] which was originally suggested for studying osmotic effects in plant cells.

At the present time we are not entirely sure that the RNA isolated by us from plant plasma sap is fully equivalent to the soluble RNA of the supernatant liquid of the homogenate. For this it would be necessary to compare both methods for the same material and to carry out supplementary physicochemical investigations of the isolated RNA. However, as the results of the present work show, the plasma sap RNA differs strongly from the RNA of the residual structures in the nature of its metabolism, and this difference resembles very much the difference between the soluble RNA and the RNA of organoids which has been demonstrated in the work of many authors.

Previous investigations showed that the plasma sap of plant leaves contained only a soluble protein which differed in its composition from the protein of organoids [19] and was a place of intensive accumulation of amino acids and peptides after nitrogen feeding [20]. Proteolytic enzymes and RNA were observed in the composition of the plasma sap of plants [21].

In the present communication we give more detailed data on the preparative isolation of RNA from leaf plasma sap and the results of a series of experiments on the study of its metabolism and function in the synthesis of intracellular proteins.

METHODS

Obtaining the Plasma Sap of Plants

The method for isolating the plasma sap from plant leaves for biochemical investigation has been briefly described in a number of studies [19, 22, 23]. Since the characteristics of this method and its use for later preparative isolation of RNA is one of the problems of the present study, we consider it necessary to give a somewhat more detailed description of the technique of obtaining plasma sap.

For obtaining the sap under pressure practically any kind of hydraulic press is suitable, one which is further equipped with pistons of various sizes. The capacity of the inner steel porous cylinder which holds the leaves varied depending on the type of press. In our work we used the simplest small hydraulic press of the school type and generated a pressure of 4000 kg on the surface of the piston; we used a cylinder with a volume of 250-300 cm^3. The plant leaves placed in this cylinder were first densely packed in a bag of strong linen and only after this were they submitted to the action of slowly increasing pressure.

Mason and Phillips as early as 1939 [18] observed that even at very high pressures only pure vacuolar sap was removed from fresh leaves while more than 50% of the liquid content of the leaves remained in the composition of the plasma sap of the almost undamaged cytoplasm. This plasma sap was easily removed from the cells, even at low pressures, on the order of several atmospheres, only after the tissue had been kept at -15 to -16°. For more complete removal of the plasma sap it was necessary to increase the pressure gradually (in our experiments, to 150-200 atm). The plasma sap was brown or yellow, and was usually clear at the moment of its extraction.

The separation of the plasma solution from the organoids was carried out in this case by ultrafiltration of the cytoplasm under pressure by removal of numerous cellulose membranes, cells, and epidermis which remained mainly undestroyed. According to the microscopic observations of Bugaevskii [24, 25] freezing and the resultant formation of ice crystals breaks up the internal structure of the protoplasts and the fine protoplasmic membranes, while the strong and thick cellulose cell walls remain unharmed. It should be noted that in the separation of soluble RNA from leaves the procedure for isolation of vacuolar and plasma sap of the cells can be combined in a number of cases, which hastens the whole analysis. In this case the fresh leaves are frozen directly and the mixture of plasma and vacuolar sap is removed from them in the press. The isolated sap also serves for separation of soluble RNA.

Isolation of RNA from Plasma Sap and Residual Structures of Plant Leaves

The following isolation of RNA from plasma sap and from residual structures was carried out chiefly by phenolic deproteinization of the material by the method of Kirbi [26], after some preliminary procedures. At first in most experiments, the protein and nucleic acid were precipitated in the plasma sap by double or triple the volume of ethyl alcohol. The precipitate obtained after keeping over night with alcohol in the refrigerator was separated by centrifuging and washed with 65-70% alcohol to remove soluble compounds.

In separation of RNA from structural residues after removal of the sap at 150-200 atm, the residues were homogenized in 0.25-0.30 M KCl, washed through a double layer of gauze and separated by centrifuging from nuclei and cell remnants for 2-3 min at 100 × g. The protein and nucleic acids of the supernatant liquid were precipitated by alcohol in the cold and the further procedures were carried out in the same way as with the RNA isolated from plasma sap.

The precipitate, washed with 70% alcohol, was treated in different ways, depending on the problems of analysis. In simple quantitative determination of RNA, the precipitate was washed with cold TCA or perchloric acid for more complete removal of acid soluble compounds, and then with alcohol, acetone, and ether to remove lipids. The dried and defatted precipitate was then hydrolyzed by perchloric acid for 20 min at 100° and after centrifuging the nucleic acids were determined spectroscopically with an SF-4 spectrophotometer by the method of Spirin [27]. When there was need for preparative isolation of RNA alone, the precipitate was suspended in water and submitted to repeated phenol treatments. In most of the other experiments, before phenol treatment, the RNA, was first extracted from the alcohol precipitate with 10% NaCl at 100° twice for three hours. Most of the protein was thus denatured and could be separated by centrifuging. The RNA and part of the protein dissolved in the 10% NaCl were precipitated by alcohol and the resulting precipitate, after washing with 65% alcohol was again dissolved in 40-50 ml of water and submitted to phenol deproteinization in the cold. The phenol treatment of the solution was carried out in a homogenizer and was repeated until complete disappearance of the film of denatured protein at the phase separation sufrace. The proteins precipitated by phenol were collected as an

independent fraction. In most cases each sample was treated with phenol (with addition of 10% chloroform) 4-6 times. Then the water layer was drawn off, freed from phenol in a separatory funnel by ether extraction and the content of nucleic acid precipitated with three times the volume of alcohol. The precipitate was reprecipitated and kept under alcohol for further determinations.

Besides RNA the precipitate contained a considerable quantity of admixtures (by weight) of unidentified polysaccharides and peptide products bound with RNA. The precipitate did not give a biuret reaction. The content of RNA in the precipitate was determined after its hydrolysis by half normal $HClO_3$ with an SF-4 spectrophotometer.

It was observed that if the precipitate obtained from plasma sap or homogenate of bean leaf structures by alcoholic precipitation was washed free of all the soluble compounds with 65-70% alcohol, then as a result of the later treatment with 10% NaCl at 100° for six hours, some quantity of peptide and polynucleotide products dissolved along with the RNA and a small amount of protein. After precipitation of the RNA and protein by alcohol, these products could easily be separated from NaCl by dialysis through cellophane. The quantitative determination of these products (calculated as sum of amino acids) was carried out after hydrolysis and chromatographic separation of the amino acids.

Determination of the Radioactivity of RNA and Proteins

In carrying out this work we particularly studied the rate of uptake of P^{32} in the different nucleic acid fractions and the rate of uptake of S^{35}-methionine in the different protein fractions and in the amino acids and peptides bound with the RNA. For more complete separation of the soluble labeled compounds from the isolated RNA and proteins at different stages of washing the preparations (at the time of salt extraction, on dialysis, reprecipitation, etc.) we always added an excess of the corresponding unlabeled carrier (phosphates, methionine and cystine, sulfates). The specific radioactivity (SA) of the preparations labeled with sulfur which had different weight and hence differing degrees of absorption of soft β-rays in a layer of the substance, were calculated according to the empirically established curve of change in activity of the precipitate spread on targets of the same diameter (18 mm), depending on the weight of the precipitate (from 1 to 50 mg). Determination of the radioactivity was carried out with an end counter T-BFL-25.

RESULTS

Comparative Determinations of Content of RNA in Plasma Sap and in Structures and the Rate of Uptake of P^{32} in the RNA of Both Cell Fractions

In recent years, but before the discovery of the characteristics of the soluble RNA it was observed in a series of papers dealing with animal materials that the RNA of cell hyaloplasm was renewed with greater rapidity than the RNA of cell structures. Shigeura and Chargaff [5] have recently carried out a number of very graphic experiments which show that the rate of metabolism of the phosphate group of soluble RNA of the liver somewhat exceeds the rate of renewal of these groups in the polymeric RNA obtained from the microsomes. Five minutes after injecting a rat with labeled phosphate preparation the soluble RNA in their experiments had almost 100 times more specific activity than the preparations of microsome RNA. This difference decreased with time and after 48 hours had almost disappeared.

As the material for the study of comparative rate of uptake of P^{32} in the RNA of plasma sap and total cell structures (microsomes, mitochondria and plastids) we used in our experiments month old bean plants. The plants were grown in 6-liter vessels in water culture of Hellriegel mixture, which was replaced by distilled water before introducing P^{32} into the vessel. P^{32} as sodium phosphate was added at 500 μC per vessel (SA 0.7 μC per mg P). Uptake of labeled phosphorus by the plant was continued for 3 hours, after which the roots were washed and the plants were replaced in the original nutritive solution. Samples of leaves (upper–young, and lower–mature) were taken, 3, 7, and 70 hours after introduction of P^{32}. Quantitative determination of RNA (in μg/g of wet weight of leaves) in the plasma sap and structures was carried out in these experiments without using phenol deproteinization of the preparations. The results of these experiments were as follows (average of determinations in three successive samples):

Mature leaves		Young leaves	
plasma sap	structures	plasma sap	structures
14.8	326	39.5	372

In the figure we show the comparative rates of uptake of P^{32} in these RNA fractions. At the same time, we measured in the experiments the radioactivity of the acid soluble phosphates of the structures and plasma sap. The radioactivity of these compounds several hours after transferring the plants to the original nutritive mixture began to fall rapidly.

In estimating the results of these experiments we can observe that the RNA content in the plasma sap of young leaves was considerably higher than in the more mature ones. The rate of P^{32} uptake in the RNA composition of plasma sap was higher than in the structure RNA, as would be expected on the basis of data already known in this respect.

Analogous data on the more rapid uptake of P^{32} in the RNA of plasma sap was also obtained in experiments with vacuum infiltration of labeled phosphorus in bean leaves and later analysis of RNA preparations isolated by phenol deproteinization.

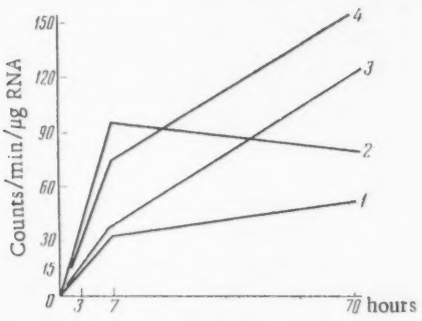
In one of the typical experiments for infiltration we used 100 g of leaves from the second and third levels

on the stem of 45 day old plants. $Na_2HP^{32}O_4$ with SA 0.5 mC/mg P was infiltrated into the leaves as a solution which contained 1 μC/ml. After the leaves had been washed with running water they were exposed to light at 17-18°. Determination of the SA of different fractions of leaf RNA two hours after infiltration showed that for plasma sap RNA this was 426 and for structure RNA, 28 counts/min/mg RNA.

During these studies we observed that successive multistage purification of RNA (extraction by hot 10% NaCl, precipitation by alcohol, and solution in distilled water, many intense suspensions with phenol and later reprecipitation) led to a rather great loss of RNA and was not suitable for quantitative determination of total RNA content in the plant cells. Part of the RNA remained with the protein, precipitating with the hot NaCl solution; part of the RNA which dissolved in 10% NaCl later did not dissolve in water, and, finally, a considerable amount of RNA was lost in phenol treatment with denatured protein. The final RNA preparations, freed from protein, (especially the RNA of the structures), was usually considerably less than half of the amount of RNA which could be found in the cells by the usual method of analysis.

One experiment on determination of the SA of RNA of plasma sap and structures was carried out with oat shoots grown in a cuvette with quartz sand. Two hours after introduction of labeled phosphate into the cuvette the SA of plasma sap RNA which was precipitated from alcohol after phenol deproteinization was more than 100 times greater than the specific activity of structure RNA, but the loss of soluble RNA in this preparative isolation was so considerable in these experiments that here we obtained very great differences in specific activity of RNA of plasma sap and structures, which could be the result of some supplementary phenomenon.

At any rate, these experiments clearly show the difference in RNA isolated from plant plasma sap and from the total residual structures (except nuclei) in the rate of their renewal.



The P^{32} uptake in the RNA of different fractions. 1) RNA of structures of lower leaves; 2) RNA of plasma sap of lower leaves; 3) RNA of structures of young upper leaves; 4) RNA of plasma sap of young upper leaves

Experiments on Uptake of S^{35}-Methionine in the Composition of Amino Acids and Peptide Products Bound with RNA, and the Composition of Different Protein Fractions

In many papers mentioned in the first part of the present work it was established that after introduction of labeled amino acids into the organism or into the incubation medium in experiments in vitro, they were very rapidly included in the composition of the soluble RNA, bound to its terminal groups. In connection with this, the SA of the soluble RNA fraction quickly increased and in the first samples considerably surpassed the SA of proteins. At later time intervals it was found that there was a transfer of radioactivity to the protein and a continuous increase in its activity. It was therefore of interest to study the dynamics of the SA in different protein and nucleic acid fractions after introduction of labeled amino acids into the plant.

The first experiments were carried out with 12-13 day old oat shoots, grown on quartz sand in a large cuvette (40 × 40 cm). Radioactive methionine (SA 0.5 mC / mg of amino acid) was added as a solution in the zone of the root system (at the root layer), using about 2 mC for each cuvette. For the preparative analysis we used a rather large sample of the green mass (300 to 400 g) so as to obtain enough plasma sap. In Table 1 we give the results of the first of these experiments in which deproteinization of the products from plasma sap and homogenates of structures, precipitated by alcohol, was carried out with phenol without preliminary extraction of the RNA by 10% Na Cl at 100°. In these experiments we determined in addition the radioactivity of the products split from RNA by weak alkaline hydrolysis (at pH 10), which, according to the data of Hoagland [6] results in the splitting from soluble RNA of the liver of about 50% of the amino acids bound to it, without destroying the polynucleotide chain.

During the process of purification, the RNA of the structures was almost completely freed from radioactive amino acid, while the RNA preparation from plasma sap had a very great radioactivity (Table 1).

A second analogous experiment was carried out to test the possibility of explaining these results by some sort of nonspecific reaction of binding S^{35}-methionine. For this purpose we introduced into the zone of the root system of the shoots not S^{35}-methionine, but $Na_2S^{35}O_4$ (SA 1 mg S equal to 10 mC). We placed 3 mC of preparation in each cuvette (in this case in protein synthesis the same amino acids were taken up as were synthesized directly in the plant leaves). Determination of the SA of the preparations of protein and RNA in the oat shoot leaves six hours after exposure showed the following (SA in counts/min/2 mg) [next page]:

Preparation	Plasma sap	Intracellular structures
Total protein, precipitated by 10% NaCl at 100°	320	306
Water insoluble fraction of salt extract, precipitated by alcohol	2232	334
Final alcohol precipitate of RNA after Phenol deproteinization of products soluble in 10% Na Cl and in water	57690	397

The results obtained in these experiments were somewhat unexpected. In both cases quantitative determination showed that the final precipitate of plasma sap contained comparatively little RNA and comparatively large amounts of products which did not give the biuret reaction and were not precipitated by TCA. In the last of these experiments we obtained about 50 mg of this precipitate from 250 ml of plasma sap. It is interesting to note that the percent content of RNA in the water insoluble precipitate of the salt extract (about 10 mg) was higher than in the final precipitate, while its radioactivity was much lower. A considerable part of the alcohol precipitate after phenol deproteinization consisted of polysaccharides. As a result of treatment of the precipitate with ribonuclease (in the second experiment) only a very small part of the radioactive product lost its ability to be precipitated by alcohol. We did not find characteristic amino acid spots in chromatography of the water solution of this fraction. After acid hydrolysis of the precipitate chromatography showed a number of amino acids, but the sulfoamino acids after such hydrolysis are usually fully destroyed in the presence of excess polysaccharides, and all the radioactivity of the hydrolyzate is found not far from the original spot of the chromatogram (when protein and peptide products are hydrolyzed in the presence of carbohydrates there is destruction and interaction of many amino acids [28] and therefore such hydrolysis is suitable only for purposes of orientation).

It is interesting to note that in the products split from RNA in weak soda hydrolysis (Table 1) it is possible to establish the presence of free amino acids only after later acid hydrolysis.

As to the origin of the final precipitate, which has maximum radioactivity, it should be noted that the radioactive complex in this case is some kind of a peptide and polypeptide product, perhaps complexed with carbohydrates and polynucleotides, and which only accompanies the soluble RNA in its isolation. Some properties of this precipitate (stability to alkaline hydrolysis, to ribonuclease, its peptide character, solubility in perchloric acid, and its great amount) show that its unusually high radioactivity is apparently determined not by the amino acids joined to the end groups of the soluble RNA molecule, but by the presence in it of some particular complex with still unclear function.

In a number of earlier works by one of the authors [29-31] on a study of the uptake of radioactivity from S^{35}-methionine and sulfates in different fractions of corn and bean proteins it was found that when the labeled compound was introduced into the root zone and the synthesis of proteins in the roots greatly surpassed the synthesis of proteins in the leaves, the conducting system transported very small amounts of polymeric, highly active soluble products, which in ordinary analysis precipitated in the fraction of vacuolar and plasma sap proteins, whose activity in this case was much greater than that of the structural proteins. In the vacuum infiltration of S^{35}-methionine directly into the leaves, these highly active admixtures were usually absent from the proteins of the sap. The localization of these compounds in the conducting and transporting systems of the leaves was established by preparative radioautographs of the leaves pressed on filter paper.

Comparing these results with those which were obtained in the present study, we feel it very probable that the final, very activie precipitate also contains some sort of peptide and polypeptide complexes, transported into the leaves from the roots along with the S^{35}-methionine and S^{35}-sulfate, and therefore it has maximum activity. This suggestion finds confirmation in the fact that with vacuum infiltration of S^{35}-methionine directly into the leaves the nature of the distribution of specific activity by fractions was somewhat different.

Vacuum infiltration of S^{35}-methionine was carried out in bean leaves. In all we carried out between July and November, 1959, three experiments which differred somewhat from each other. In the first experiment the leaves were taken from levels 2-3 of 45 day old plants; in the second and third experiments we used as material for infiltration leaves from the levels 1-2 of younger 20 day old plants. The SA of S^{35}-methionine and its concentration in the solution were somewhat different in the different experiments and therefore it was necessary to compare not the different experiments, but the activity of the fractions in each experiment separately. S^{35}-methionine had the following activities per mg: in experiment I, 0.31; in II, 0.17; in III, 0.48 MC. Also in the

TABLE 1. Radioactivity of Preparations of Proteins, RNA, and Products Split from RNA, Isolated from Oat Leaves after Exposure of the Plants to a Medium with S^{35}-Methionine (in counts/min)

Preparation and amount	Plasma sap		Intracellular structures	
	2.5 hrs*	6.0 hrs	2.5 hrs	6.0 hrs
Total protein, μg	32	58	32	163
Products split from 100 μg RNA by 20 min hydrolysis with 0.1 N $NaHCO_3$ at 37°	3280	6250	bkgd.	bkgd.
RNA after removing products split by soda hydrolysis (per 100 μg RNA)	7200	48400	10	10

*Time readings were taken at the moment of adding S^{35}-methionine in the zone of the root system. Since intensive accumulation of radioactivity began only after 1.5-2 hours from the time the experiment began, readings were not made before this period.

first experiment the leaves were exposed after infiltration in natural light at 17-18°, while in the other two experiments exposure of the leaves was carried out under daylight lamps at 24-25°. In addition, in the second experiment some determinations were not carried out because of the accidental loss of the preparation during phenol treatment. The period of time from beginning of infiltration to the freezing of the leaves in all cases was two hours. In spite of these differences the results of the experiments on comparing the activity of the preparations in each of the experiments were fully the same, and are summarized in Table 2.

S^{35}-methionine was introduced into the leaves as a solution which contained in 1 ml 19.6 and 17 μcurie respectively in the first, second and third experiments. The weight of leaves used for infiltration in these experiments was 100, 200, and 300 g. It is known that in vacuum infiltration in leaves it is possible to "inject" about 10-12% of the solution with respect to the initial wet weight of the leaves, and therefore for each 10 g of wet weight we used about 6 to 20 μcurie of radioactive amino acid.

As the same time as the determination of SA we determined the content of polynucleotides in these fractions. The maximum quantity of polynucleotides was found in the final RNA precipitate; however, in nondialyzed fraction (3) the precipitate of which in both experiments had maximum SA, we also found a considerable amount of polynucleotides (about 10-20% of the amount found in the final RNA precipitate).

The presence of polysaccharide contaminants in the final RNA precipitate certainly decreased its actual SA, as could be calculated, for example, on the weight of methionine in these products. The chromatographic determination of methionine in the RNA preparations was very difficult, since it was decomposed and oxidized by hydrolysis in the presence of carbohydrates and RNA.* Chromatograms of the hydrolyzate from the final RNA precipitate of plasma sap are therefore only orienting, but on these chromatograms we can clearly find the chief amino acids (cystine, lysine, glycine, serine, aspartic and glutamic acids, alanine, phenylalanine, leucine, isoleucine, and less clearly, some others). On the chromatogram of the nondialyzed peptide products of the plasma sap and structures are clearly shown all the amino acids which could be identified also in the proteine.

DISCUSSION

If we refrain for the present from analysis of the material obtained in the experiments with introduction of labeled S^{35}-methionine and sulfates in oat plants, some peculiarities of which are evidently connected with the presence of some very active compound from the conducting system, then the data of the last table can also be considered as preliminary. In these experiments we noted in the cell protoplasm a fraction (fraction 3, Table 2) which in the usual schemes of preparative analysis, in experiments for determining the rate of synthesis, is as a rule discarded along with the supernatant liquid after salt extraction, or else it enters the composition of the total protein in less detailed treatment. The polypeptide and polynucleotide products which enter its composition, insoluble in 60-70% alcohol before deproteinization of the plasma sap and structures also pass into the soluble state (in 70% alcohol) after treatment of the precipitate with 10% NaCl at 100°. This permits us to suggest that the above compounds, at first loosely bound in the protoplasm by some sort of structure, enter the free state when these bonds are destroyed. These same compounds, separated from the

*The amino acids labeled with C^{14} in the carboxyl group, which we had at our disposal, were still less suitable for this study, since they were very quickly decarboxylated in plant tissues; as is known, plants then easily assimilate the evolved carbon dioxide directly in their tissues.

TABLE 2. Radioactivity of Different Fractions Isolated from Bean Leaves Two Hours after Infiltration into the Leaves of S^{35}-methionine, counts/min/2 mg

Fraction	Experiment I		Experiment II		Experiment III	
	plasma sap	structure	plasma sap	structure	plasma sap	structure
Total protein (1)	1788	1514	2982	2886	3364	2647
Precipitate obtained in dialysis of product soluble in 10% NaCl at 100°, but not precipitated by alcohol (2)	not det.	not det.	7116	3150	6420	4782
Polypeptides and peptides separated from the total alcoholic precipitate after its treatment with 10% NaCl at 100°, nondialyzing and not precipitated by alcohol (3)	not det.	not det.	13244	4180	7463	778
RNA + protein before phenol treatment (4)	746	404	not det.*	1300	1938	356
Final alcohol precipitate of RNA (5)	364	72	not det.	64	1743	19

*Preparation was lost at the time of analysis.

plasma sap, have the maximum SA, greatly surpassing the SA of total protein. The SA of the amino acids bound to the terminal groups of the RNA is perhaps higher, but this has not yet been firmly established. We have not carried out short term expriments to determine the SA of the polynucleotides of this fraction in experiments with P^{32}.

Only one experiment not considered in the given report was carried out in which we considered the activity of all the fractions three days after introduction of P^{32} in the root system zones of beans. With such exposure the different values of SA of the preparations strongly leveled off, but in this case the polynucleotides of these fractions had great activity compared to the activity of RNA isolated from structures.

The polypeptide products contained in these fractions were about 4-6 times higher (by mass) than the content of polynucleotides, but in calculations by mass of the total protein they made up only a few percent. It was possible to isolate a considerably greater mass of these products from the structures, calculated on wet weight of the leaves, than from the plasma sap (by 3-4 fold), although the SA of these components was considerably lower in the structures.

The functional role of this fraction is still not very clear. However, it is interesting to note that although, according to present ideas, molecules of RNA of the structure are matrices for protein synthesis, and hence should contain at the moment of analysis some incompleted peptide chains in fixed positions, yet actually the RNA of the structures are almost completely free of labeled methionine. The RNA of plasma sap is quite different in this respect: its bond with labeled S^{35} compounds is considerably stronger. The presence in the composition of RNA, deproteinized by phenol, of amino acids, peptides, and protein complexes was noted in the work of a number of authors [32-35]. However, in purely analytical work it always remains uncertain whether these are products intermediate in protein synthesis or only the result of incomplete deproteinization, or in any way can be secondary forms of chemical reaction with RNA. The rather high percent of these admixtures in the analysis of total RNA from oat shoots [34] could, for example, be the result of some soluble complex of root origin which accompanies it in the plasma sap, as is indirectly indicated by the results of our work.

In the recent work of Dintzis, Borsook, and Vinograd [35] in the microsomes of reticulocytes were found polypeptides and peptides extractable by heat, which in experiments with labeled amino acids had a very high activity and considerably more rapid renewal than the total protein. Only in the cells of Pseudomonas hydrophila was there a detailed study of Connell and Watson [37] of the variety in composition of the peptide fractions formed as a result of synthetic processes which proceeded protein synthesis.

Numerous facts as to the share of peptides in the synthesis of proteins have also been given in a recent review devoted to the biological role of the peptide fractions [38]. Based on these materials we can express the following ideas of the nature of peptides and polypeptides with maximum activity in the plasma sap and less activity in the peptide fraction of the structures.

Evidently, in spite of the participation of the molecule of soluble RNA in protein synthesis as a specific adaptor, the synthesis of polymeric protein molecules occurs stepwise, with intermediate formation of peptide and polypeptide products, loosely bound to the matrix. The peptide fraction of the structures, separated from them after treatment with hot 10% NaCl evidently consists of incomplete protein products of the primary and secondary cycles of their enlargement, while the analogous peptide fraction of plasma sap consists of intermediate peptides which are evidently formed on the matrix but then are split off for new uptake into the composition of some structure for further "assembling" of the protein molecule.

SUMMARY

The plasma sap of frozen plant tissues, separated by pressure and forming an ultrafiltrate of cell cytoplasm is a suitable and easily available fraction for preparative isolation of large amounts of RNA. This RNA is similar in some respects to the RNA found previously only in the supernatant fraction after precipitation of the microsomes by rapid centrifuging.

The plasma sap RNA isolated from bean and oat leaves has a considerably greater rate of phosphorus metabolism than the RNA of the total structural elements.

In experiments with uptake of S^{35}-methionine in the composition of cell components it was found that the plasma sap RNA of cells after its deproteinization always contained a considerable amount of radioactivity due to the presence of amino acid-peptide products, while the structural RNA could be almost completely freed from labeled S^{35} products.

In leaf protoplasm a fraction was found which consisted of polypeptides and polynucleotides which was separated from the total alcohol precipitate of plasma sap and structure homogenate by treatment with 10% NaCl at 100°. These compounds were not precipitated by alcohol, which permits their separation from the nucleic acids and proteins. The products contained in this fraction from plasma sap had maximum radioactivity.

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THE AMINO ACID COMPOSITION OF PROTEIN-FREE EXTRACTS OF VERTEBRATE AND INVERTEBRATE SMOOTH MUSCLES

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The extractable nitrogenous compounds in the smooth muscles of vertebrates, and particularly those of invertebrates, have received very little attention. Recent communications have dealt with some of the phosphagens of smooth muscle [1], with the distribution of nitrogenous bases in muscles of lobsters and shrimp [2, 3], and with the free amino acids in the muscles of the molluscs Mytilus edulis and Mya arenaria [4]. In the latter work, 23 amino acids were identified in the protein-free extracts, but the results were of a purely qualitative nature.

The present work deals with both the qualitative and quantitative distribution of the free amino acids and peptides in the smooth muscle of the pigeon stomach and of the adductor and foot muscles of the molluscs Anodonta and Mydial.

METHODS

The required muscles of the pigeon and of the two molluscs were dissected free from surrounding tissue and ground up.

A number of protein precipitation techniques were tested. They included cold precipitation with TCA, tungstic acid, perchloric acid, or dilute acetic acid with boiling for seven minutes. The extraction procedure finally adopted was as follows: The ground tissue was homogenized with three volumes of 5 % TCA to a fine suspension. The suspension was centrifuged and the precipitate re-extracted with 5% TCA. The pooled extracts were filtered and, since those of the invertebrates (and particularly of Anodonta) were strongly opalescent, were treated with an equal volume of ethanol. The resultant precipitate was centrifuged off and washed thrice with ethanol. The pooled supernatants were evaporated on the water-bath. TCA was removed from the concentrated extracts by shaking in a separating funnel at least 10-12 times with three volumes of ether. The same standard procedure was adopted for the extracts of pigeon stomach muscle which were quite clear from the outset. The ether layers of the first three extractions were usually yellow. The color was particularly pronounced with extracts from the yellow part of the Anodonta foot. The ether layer contained fats and other fat-soluble substances, like chromogens and pigments. Towards the end of the extraction some slight precipitates of a hydrophilic nature appeared and were removed by centrifugation. The fat-free extract was evaporated on the steam-bath to about 0.5–1.0 ml (and centrifuged if necessary), and the clear solution was used for chromatography. In some cases the evaporation was carried through to dryness and the dry residue was treated with ethanol, slightly acidified with 0.1 N HCl.

Chromatographic paper (Leningrad No. 2) was washed prior to use with a 1% solution of oxine (8-hydroxyquinoline) in the solvent butanol-glacial acetic acid-water (4:1:5). The paper was then washed in the chromatography vessel by descending irrigation with the same solvent for 2-3 days, and then with water. Papers treated with versene and then copiously washed with water were also used.

Application of extracts to the paper. The papers used were 65 × 25 cm. A line parallel with the lower edge, and 7 cm distant from it, was drawn across the sheet. Several pentagons, 2 cm distant from each other, were drawn above that line. Sections of the line mentioned above, each 4 cm long, served as the bases of the pentagons. Their sides were perpendicular to the base, and drawn upwards to a length of 5 cm. The remaining two sides were drawn upwards to meet at a median point. The strips between the pentagons, 2 cm wide, were used as origins. The solutions, in volumes depending on the solute concentrations in the various tissues, were applied, each to one of those strips, equidistant from the bottom line, while other positions were occupied by reference compounds. The latter were amino acid solutions in either 10% ethanol or double-distilled water. Reference solutions contained 2 μg of the amino acid per liter, and 5-10 μg of any given amino acid was applied to reference strips.

After the application of the mixtures and the reference standards the pentagonal sections were cut out and the chromatograms were placed in the solvent. The most suitable mixture for the separation of amino acids was found to be butanol-acetic acid-water (4:1:5 or 8:3:1). The chromatograms were then developed with a 2 %

solution of ninhydrin in acetone and allowed to remain in the dark for 24 hours at room temperature. The alternative treatment - heating for 10 min at 50-60°- resulted in discoloration of the background and interfered with quantitative determinations. The developed chromatograms were stored in the cold in order to delay fading of the spots. When long storage was required, the chromatograms were fixed with a 0.5 N solution of nickel sulfate.

The protein-free extracts of the smooth muscles of vertebrates and invertebrates were checked firstly for the presence of the peptides carnosine and anserine. These were precipitated from the extracts as mercury complexes in the presence of ethanol. After suitable treatment of the crude precipitates the solutions thus obtained were applied to chromatograms. Amino acids and peptides were separated by ascending chromatography in phenol (b.p. 181°) saturated with water. On some occassions HCl vapor was introduced into the atmosphere by placing a vessel of aqueous (1:2) HCl inside the chromatography chamber.

The "phenolic" chromatograms, after removal of the solvent with hot acetone, were developed for histidine and carnosine with a diazo-reagent, and for anserine -with ninhydrin. On no occasion were the amino acid, histidine, or the peptides,carnosine and anserine, detected on chromatograms of smooth muscle extracts. *

Experimental work with the smooth muscle extracts consisted mainly of two parts: the qualitative chromatographic analysis of the amino acid composition and the quantitative estimation that followed.**

Quantitative determinations were carried out according to Giri and co-workers [5], with the modifications of Zaitseva and Tyuleneva [6]. Colorimetric measurements on the ninhydrin-copper complex at 530 mμ were made in an FEK-M colorimeter with a green filter No. 2. Colorimetric readings were translated into amino acid concentrations with the aid of corresponding standard curves.

RESULTS

The qualitative results, on the amino acid composition of the protein-free extracts of vertebrate and invertebrate smooth muscle can be seen from the reproduced chromatograms. Chromatograms 1 and 2 represent the amino acids of the protein-free extract of the pigeon stomach. The chromatogram shown on Fig. 1 represents the result of separations of amounts of extracts corresponding to 26 and 52 mg tissue, respectively; those shown in Fig. 2. were prepared from smaller applications. This was done in order to facilitate the quantitative determination of those amino acids which occur in the tissues in comparatively high concentrations, while the heavy applications allowed the determination of the minor constituents. The availability of a range of total material applied to the origin allowed the quantitative determination of all the amino acids present.

An inspection of Fig. 1 showed that, as judged from the intensity of the ninhydrin-stained spots, the amino acids ranged in their content in the extract as follows (in decreasing order of intensities): glutamic acid, glutathione, glutamine, aspartic acid, serine, glycine, alanine, arginine and leucine. Those found in smaller amounts were: cystine + cysteine, ornithine + lysine, threonine, β-alanine tyrosine and methionine + valine. It should be added that the spots marked 13, 14 and 15, and corresponding to β-alanine, tyrosine and valine + methionine, respectively, and which are poorly reproduced on the photograph, were clearly visible on the original chromatogram.

The fact that cystine and cysteine, ornithine and lysine and methionine and valine have actually been demonstrated on the chromatogram is supported by the observation that, when reference standards of those six amino acids were chromatographed together in the usual fashion in the solvent used, the three pairs mentioned failed to show separation of their two components which frequently showed up as one spot.

The qualitative amino acid composition of the protein-free extracts of the foot and adductor muscle of Anodonta is shown in Figs. 3 and 4.

The chromatogram shown on Fig. 3 represents the separation of a quantity of extract corresponding to 60 mg tissue. Glutathione and 13 amino acids are clearly discernible. The glutathione spot, which was cone-shaped and unlike that of the reference compound, was later found to be a mixture containing, in addition to glutathione, also cystine and cysteine. In order to demonstrate this point other solvents and precipitants were employed. Deproteinization with dilute acetic acid (0.04 N) with seven-minute boiling produced an extract which gave a somewhat different chromatographic pattern (Fig. 4). Here, between the spots corresponding to arginine and serine, at least two other spots (glutamine and aspartate) appeared. On the same chromatogram there appeared also an additional unidentified spot above that of tyrosine; this could be seen at all levels of application. Thus the extracts of the Anodonta muscle were found to contain various amino acids, qualitatively corresponding to those present also in the protein-free extracts of thepigeon stomach muscle.

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* Proline, histidine and other amino acids, not found in the protein-free extracts of Mydial, were easily detected in the 24-hour, 5 N HCl hydrolyzates of the protein precipitates of this species.

** The portions of the extracts used for quantitative work were extracted 10-12 times with ether; after removal of excess ether and evaporation to 1.5-2 ml the residue was treated with an equal volume of ethanol and the precipitate was centrifuged off and washed with ethanol. The pooled supernatatnts were concentrated to 0.5-1 ml and applied to chromatograms.

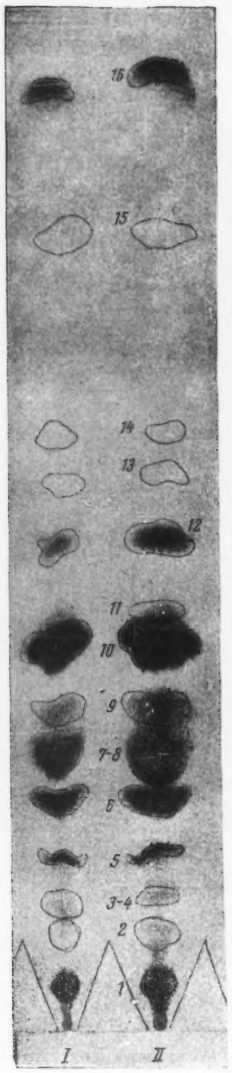

Fig. 1.

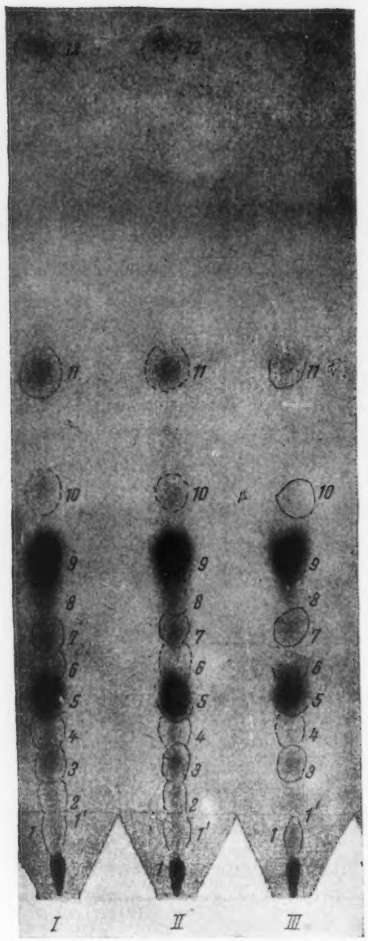

Fig. 2.

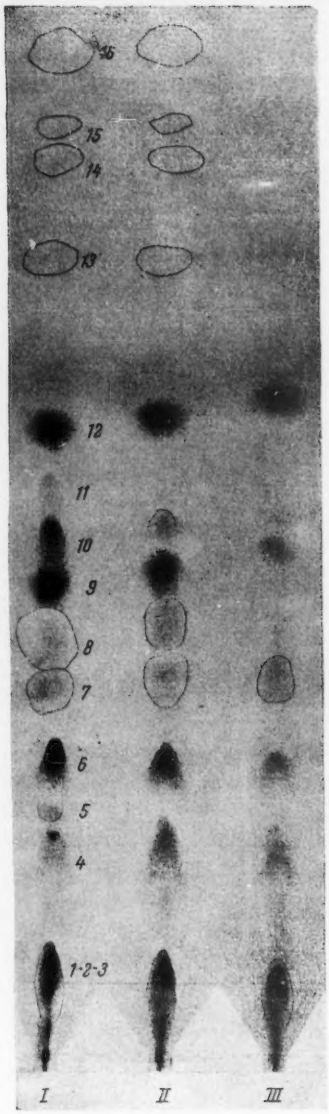

Fig. 3.

Fig. 1. Chromatogram of an extract of pigeon stomach muscle, with heavy applications of the following: I) Corresponding to 26 mg tissue; II) corresponding to 52 mg tissue. 1) glutathione; 2) cystine + cysteine;3,4) ornithine + + lysine; 5) arginine; 6) glutamine; 7) aspartic acid; 8) serine; 9) glycine; 10) glutamic acid; 11) threonine; 12) alanine; 13) β-alanine 14) tyrosine; 15) methionine + valine; 16) leucine + isoleucine.

Fig. 2. Chromatogram of an extract of pigeon stomach muscle, with light applications. I) Corresponding to 10 mg tissue; II) corresponding to 7.5 mg tissue; III) corresponding to 5 mg tissue. 1) glutathione; 1' - cystine + cysteine; 2) ornithine; 3) lysine; 4) arginine; 5) glutamine; 6) aspartic acid; 7) serine; 8) glycine; 9) glutamic acid; 10) threonine; 11) alanine; 12) leucine-isoleucine.

Fig. 3. Chromatogram of material obtained from the smooth muscles of the foot and adductor muscle of Anodonta. Amino acid distribution in extracts prepared by TCA precipitation. Amounts of extracts applied corresponded to the following amount of tissue (in mg): I) 60, II) 30; III) 15. 1,2,3) glutathione + cystine + cysteine; 4) ornithine; 5) lysine; 5) arginine; 7) serine; 8) glycine; 9) glutamic acid; 10) threonine; 11) sarcosine; 12) alanine; 13) tyrosine; 14) valine + methionine; 15) phenylalanine; 16) the leucines.

Fig. 4

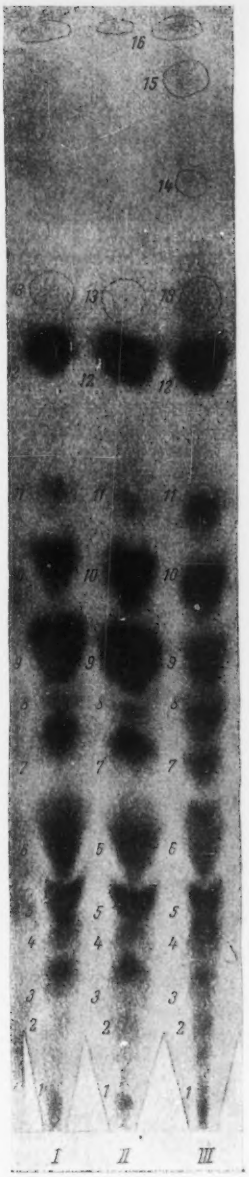

Fig. 5

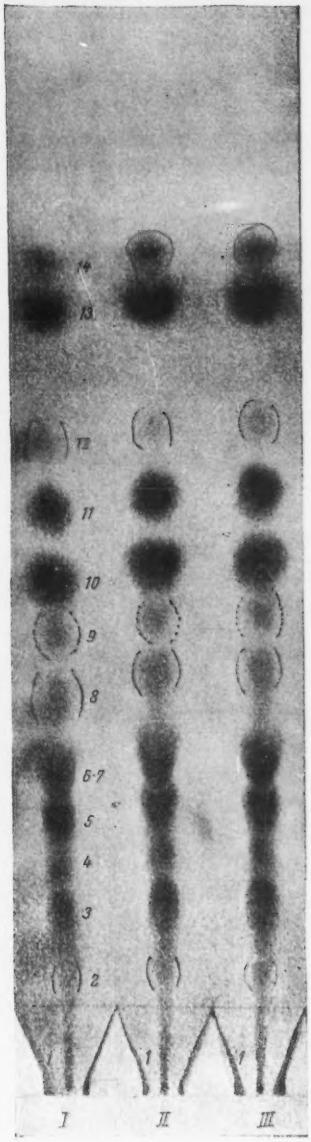

Fig. 6

Fig. 4. Chromatogram of extracts of the smooth muscle from the foot and adductor of Anodonta. Amino acid distribution in extracts obtained by deproteinization with 0.04 N acetic acid. Equivalent of tissue applied (mg): I) 15; II) 30; III) 45. 1) glutathione; 2) unidentified; 3,4) cystine + cysteine; 5,6) ornithine + lysine; 7) unidentified; 8) arginine; 9) glutamine; 10) aspartic acid; 11) serine; 12) glycine; 13) glutamic acid; 14) threonine; 15) sarcosine; 16) alanine; 17) tyrosine; 18) unidentified; 19) methionine + valine; 20) phenylalanine; 21) the leucines.

Fig. 5. Chromatograms of extracts of various tissues of the Mydial: I) Foot; II) adductor muscle; III) mantle. Amounts of extract applied corresponded to 20 mg of each tissue. 1) glutathione; 2) cystine + cysteine; 3) ornithine; 4) lysine; 5) arginine, 6) taurine + glutamine; 7) aspartic acid; 8) serine; 9) glycine; 10) glutamic acid; 11) threonine; 12) alanine; 13) β-alanine; 14) tryptophane; 15) phenylalanine; 16) the leucines.

Fig. 6. Chromatograms of extracts of the foot and adductor muscles of the Mydial. Amount of extract applied corresponded to: I) 10 mg; II) 15 mg; III) 20 mg of tissue. 1) glutathione; 2) cystine + cysteine; 3) ornithine; 4) lysine; 5) arginine; 6,7) taurine + glutamine; 8) aspartic acid; 9) serine; 10) glycine; 11) glautamic acid; 12) threonine; 13) alanine; 14) β-alanine.

TABLE 1. Free Amino Acid Composition of the Smooth Muscles of the Pigeon Stomach and of the Foot and Adductor Muscles of Anodonta and Mydial (In mg % of wet tissue weight)

Amino acid	Smooth muscle of pigeon stomach		Smooth muscle of Anodonta		Smooth muscles of Mydial	
	amino acids	nitrogen	amino acids	nitrogen	amino acids	nitrogen
Tripeptide glutathione	184,1	25,4	48,6	6,8	13,3	1,8
Cystine + cysteine	40,2	4,6	26,1	3,1	52,7	6,2
Ornithine	10,6	2,3	22,8	4,7	70,8	6,5
Lysine	12,0	2,3	19,0	3,6	42,7	8,2
Arginine	15,0	4,8	54,8	17,6	130,3	41,8
Taurine	—	—	—	—	109,6	12,4
Glutamine	128,4	25,2	13,7	2,6	24,3	4,6
Aspartic acid	24,5	2,7	17,3	1,8	70,0	7,3
Serine	26,8	3,4	15,2	2,0	40,2	5,4
Glycine	20,3	3,8	19,7	3,7	263,0	48,9
Glutamic acid	211,2	20,1	32,0	3,0	95,1	9,4
Threonine	6,9	0,9	3,5	0,5	21,9	3,0
α-Alanine	24,3	4,5	46,8	7,5	139,5	22,2
β-Alanine	1,8	0,3	0,6	0,1	23,7	3,8
Tyrosine	+		+		+	
Tryptophane	+		+		+	
Methionine + Valine	+		+		+	
Phenylalanine	8,0	0,6	11,7	0,9	7,0	0,6
Leucine	13,3	1,4	14,2	1,5	22,8	1,8
Isoleucine	+		+		+	
Total	727,4	102,3	346,0	59,5	1126,9	183,9

The chromatograms of the free amino acids in the extracts of the adductor, mantle and foot muscle of Mydial are presented in Figs. 5 and 6.

It will be seen from Fig. 5 that the extracts of the foot and adductor muscles did not differ in their amino acid composition. The mantle of the Mydial was found to contain the same complement of free amino acids as the smooth muscle, but the quantitative distribution was different. When chromatograms, corresponding to the same amount of tissue (20 mg), are compared, the intensity of the ninhydrin colors of some of the amino acids can be seen to differ, depending on the source; thus threonine, serine, cystine and lysine, and to a lesser degree also glycine, arginine, aspartic acid and taurine, are seen to occur in the mantle in higher concentrations than in either adductor or foot muscles. The remaining amino acids are distributed comparatively uniformly amongst the three tissues investigated.

The muscles of Mydial, unlike those of the Anodonta and the pigeon stomach, contain large amounts of taurine, which in the chromatographic system used runs together with glutamine. This has in the past led to an erroneous assumption that the tissues of Mydial contained no free glutamine. However, further resolution (by electrophoresis) of eluates of the taurine spots showed the presence of glutamine also: the latter accounts for about one-fifth of the total compounds in the "taurine" spot. By the same method, a small taurine contaminant was also shown to be present in the arginine spot. These three compounds can therefore not be satisfactorily resolved by a single chromatographic step.

The presence of the following 16 amino acids was demonstrated in the foot and adductor muscles of the Mydial: cystine + cysteine, ornithine, lysine, arginine, aspartic acid, serine, glycine, glutamic acid, threonine, sarcosine (in small amounts), alanine, β-alanine, tyrosine, tryptophane, phenylalanine, leucine + isoleucine, glutathione, glutamine and taurine.

The quantitative distribution of the free amino acids in the various tissues investigated is shown in Table 1.

It follows from the Table that the smooth muscles of both vertebrate and invertebrate origin contained the same complement of free amino acids. The only exception to this rule was taurine, which was found, in rather large amounts, in the Mydial alone.

On the other hand, the quantitative distribution follows different individual patterns in the species investigated. The total non-protein amino acids in the Mydial (1127 mg %) is three times that found in Anodonta (346 mg%) and 1,5 times that of the pigeon stomach wall (728 mg%). The major nitrogenous, non-protein components of the pigeon stomach wall are glutamic acid, glutathione and glutamine; in the Mydial they are glycine, alanine, arginine and taruine; in the Anodonta - arginine, alanine and, with reservations, glutathione, since the values for glutathione nitrogen (Fig. 4) represent a sum of ninhydrin-reacting compounds. The remaining amino acids-tyrosine, tryptophane, methionine, valine - were not determined quantitatively since, although their presence in the extracts was beyond doubt, their concentrations were low.

DISCUSSION

The present results demonstrated that in the tissues studied - the smooth muscles of the pigeon stomach and the muscles of Anodonta and Mydial- the general amino acid make-up showed only slight differences, although their quantitative distribution showed considerable variation. The highest amount of free amino acids was found in the marine form of Mydial - 1127 mg% compared to 346 mg% in the fresh-water Anodonta. This three-fold difference between the two species could be due to the different habitat. For instance. Florkin [7] reporting the high free amino acid concentrations in marine crustacea, compared with fresh-water crustacea, considered the raised levels to be a response reaction to the high osmotic concentration of the sea water. According to our data [8], the total extractable nitrgoen in the Mydial amounted to 550 mg% in the foot muscle and 525 mg% in the adductor muscle. About 40% of this nitrogen is accounted for by amino acids. In the Anodonta the total extractable nitrogen amounted to 202.7 mg% and 158.2 mg% in the foot and adductor muscles, respectively. Of this value, only about 25% is amino acid nitrogen. This large difference in the amino acid content of marine and fresh-water forms can also be due to differences in nutritional conditions and habits.

Thus the complement of free amino acids present in the various vertebrate and invertebrate muscles, as well as the prevalence of certain amino acids in some forms, appear to be far from accidental.

Glutamic acid, glutathione and glutamine appear to be the prevalent amino acids in the vertebrate smooth muscle (pigeon stomach wall). On the other hand, in the invertebrates glycine, alanine, arginine, taurine and glutamic acid play an important role in the composition of muscle free amino acids. They are found in those tissues in high concentrations. It is well known that in the muscles of some invertebrates arginine, in the form of the phosphagen phosphorylarginine, plays a very important role, comparable with that of phosphorylcreatine in vertebrate muscle.

SUMMARY

The qualitative amino acid composition of protein-free extracts of vertebrate smooth muscle was found to be essentially the same as that of invertebrate smooth muscle. The only exception was found to be taurine, present in the Mydial, but absent from the Anodonta and from the pigeon stomach wall.

The marine forms of Mydial contain approximately three times the concentration of free amino acids than the fresh-water Anodonta and about one-and-one-half times that of the smooth muscle of the pigeon stomach.

The dipeptides, carnosine and anserine could not be detected in the smooth muscles of either vertebrates or invertebrates, but muscle of all species tested was found to contain the tripeptide glutathione, which is present in particularly high concentrations in the muscles of the pigeon stomach wall.

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THE EFFECT OF PROLONGED DAILY IRRADIATION ON NUCLEIC ACID TURNOVER IN SOME TISSUES OF THE RABBIT

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Numerous investigations [1-5] have dealt with the effect of a single dose of ionizing radiation on animal metabolism, but the works dealing with the effect of chronic irradiation are very few. In view of the fact that nucleic acids are considered among the most radiosensitive compounds within the living cell, and since nucleic acids are intimately concerned with the processes of division, regeneration and the possibility of reconstitution, it was considered of some interest to study the metabolism of these compounds in the most radiosensitive (bone marrow) and a relatively radioresistant (liver) tissue of rabbits subjected to chronic total body radiation (TBR).

METHODS

A total of 33 rabbits of both sexes, weighing 2.5-3.0 kg, were given daily (six days per week) doses of 30 r TBR. The γ-radiation source (Co^{60}) delivered the radiation at a rate of 0.7 r / min. The animals were killed by air embolism after they had received total doses of 210, 420, 630, 900, 1410, 1920 or 2490 r, corresponding to 7, 14, 21, 30, 47, 64 and 83 days' irradiation, respectively. Each irradiation group contained four rabbits, the control group - five. Nucleic acids (NA) in liver and bone marrow were determined according to Schmidt and Thannhauser, with the modification by Davidson [6] Phosphorus incorporation was measured four hours after the injection of 60 μC of $Na_2HP^{32}O_4$ per kg body weight.

The mean nucleic acid content per cell was calculated from the known analytical values by counting the number of cell nuclei in one g of tissue. The results were also evaluated on the basis of differential cell counts in bone-marrow smears and mitotic counts in liver sections.

Relative specific activities (RSA) were calculated from the determined values of the SA of various acid-soluble phosphorus compounds as described earlier [7]. All results were expressed as the arithmetical mean of the determinations with the error of the mean.

RESULTS

Daily irradiation with 30 r caused some loss of weight in the rabbits and, from the second week of treatment onwards, a fall of the leucocyte and thrombocyte count in the blood. The red blood cell (RBC) count and total hemoglobin were unaltered.

It can be seen from Table 1 that there were no appreciable changes in RNA-P and DNA-P content of liver. The DNA-P content (per g tissue) fell in the initial stages of irradiation (at the 420 r level) by 24%. Continuing irradiation caused a slight increase in the DNA-P content, and its value returned to normal level after a total dose of 2490 r (83 irradiations). At total dose levels of 210 and 900 r RNA-P levels fell slightly (by about 20%). At all other TBR levels the RNA-P -content was either normal or slightly above that.

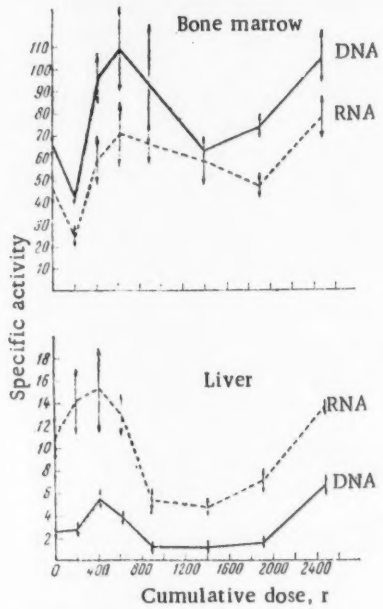

Fig. 1. The specific activities of the RNA and DNA phosphorus of liver and bone-marrow in animals receiving different cumulative doses of TBR. (Animals were given 60μC of $Na_2HP^{32}O_4$ per kg body weight and killed four hours later.)

TABLE 1. Nucleic Acid Content in Liver and Bone-Marrow of Rabbits at Various Stages of Chronic Irradiation.

Dose, r	Liver wt. / body wt.	NA per g tissue		RNA-P / DNA-P	Cell count per g × 10^8	NA per cell	
		RNA-P, mg	DNA-P, mg			RNA-P × × mg^{-10}	DNA-P × × mg^{-10}
Liver							
Control	0,035±0,002	0,78±0,04	0,25±0,002	3,21±0,30	1,62±0,12	46,7±4,0	13,3±1,4
210	0,041±0,004	0,62±0,01	0,17±0,06	3,81±0,41	1,62±0,10	38,6±2,3	10,3±0,8
420	0,035±0,003	0,97±0,08	0,19±0,02	5,00±0,21	1,47±0,12	66,5±4,3	13,4±1,1
630	0,029±0,003	0,92±0,04	0,23±0,03	4,17±0,57	1,31±0,003	70,8±6,1	17,8±2,2
900	0,037±0,002	0,61±0,01	0,32±0,03	1,95±0,18	1,70±0,04	35,9±0,8	18,8±1,5
1410	0,031±0,001	0,87±0,07	0,32±0,05	2,75±0,23	1,65±0,08	53,4±7,0	20,1±3,9
1920	0,024±0,001	0,90±0,03	0,31±0,01	2,83±0,11	1,84±0,04	47,7±1,8	16,9±0,2
2490	0,026±0,001	0,79±0,09	0,22±0,02	3,64±0,27	1,77±0,21	46,2±7,3	12,8±2,0
Bone marrow							
Control	—	0,32±0,04	0,56±0,07	0,58±0,06	6,18±0,97	5,3±0,5	9,2±0,5
210	—	0,48±0,11	0,50±0,07	0,93±0,11	4,86±0,09	9,6±1,1	10,4±0,2
420	—	0,35±0,04	0,48±0,05	0,75±0,07	4,79±0,49	7,2±0,2	10,1±1,2
630	—	0,26±0,02	0,39±0,08	0,70±0,07	4,38±0,54	6,3±0,7	9,2±1,1
900	—	0,19±0,02	0,37±0,03	0,51±0,03	2,38±0,24	8,2±1,0	15,8±1,1
1410	—	0,24±0,03	0,33±0,02	0,72±0,01	2,01±0,10	12,0±1,1	16,7±1,1
1920	—	0,18±0,02	0,29±0,01	0,62±0,03	3,02±0,25	6,1±0,1	9,9±0,4
2490	—	0,25±0,05	0,40±0,08	0,63±0,03	3,97±0,90	6,1±0,8	10,3±0,4

An investigation into the incorporation of P^{32} into the NA (Fig. 1) showed that a comparatively small (for a rabbit) cumulative dose of 420 r (14 × 30 r) increased the SA of DNA-P by a factor of 2.2. Further irradiation brought about the decrease of this value until, at a cumulative dose of 900 r (30 irradiations), the SA of DNA-P was only 26.4 % of control value. When the cumulative dose reached 1400 and 1920 r the fall in SA of DNA-P was less marked, while at 2490 r it again increased to about 2.7 times the control value. The dynamics of the change in SA of RNA-P was similar to that of DNA-P, but the intensity of the changes was less pronounced.

A study of the RSA values (Fig. 2) showed that the changes in P^{32} incorporation observed actually reflected true increases or decreases in the turnover of the NA, and were not due to altered permeability of the cells for phosphate.

Nuclear count per g of liver revealed that prolonged irradiation caused no significant changes in cell numbers. Thus, at a cumulative dose of 630 r, there was a detectable fall in nuclear count (by 19%). Further irradiation tended to level out the early effect, and sometimes to stimulate even excess cell division, as evidenced by a slight increase (13%) of the nuclear count over control value at the 1920 r level. The mean DNA-P content per liver nucleus was elevated at most stages of irradiation. The highest DNA content per nucleus (about 1.5 times control value) was observed in animals given a cumulative dose of 1410 r (47 irradiations). The mean RNA content per nucleus was also variable. The maximal levels (about 1.6 times the control value) were found after a cumulative dose of 630 r (21 irradiations).

Seven irradiation periods of 30 r each failed to affect the NA content in bone marrow. Further irradiation caused an appreciable fall of the NA content per g tissue. Thus, when the cumulative dose of 900 r (30 exposures) was given, the RNA-P and DNA-P content of bone marrow was considerably below normal values (60% of controls).

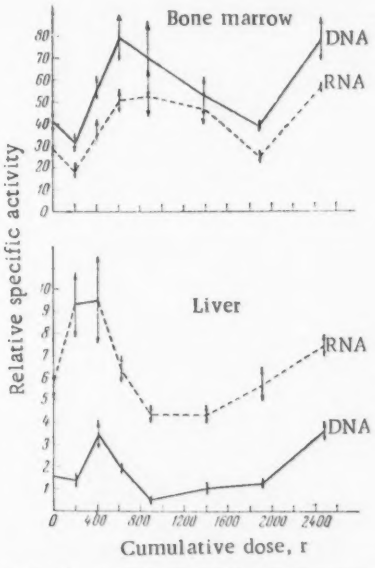

Fig. 2. Relative specific radioactivity of the RNA and DNA phosphorus of liver and bone-marrow in animals receiving different cumulative doses of TBR.

Two and one half months from the beginning of the treatment (cumulative dose, 1920 r - 64 irradiations) these values fell further to 58% and 53% of control values for the RNA-P and DNA-P, respectively. A slight rise of the nucleic acid content was then observed at the 2490 r level.

The specific activity (SA) of the RNA-phosphorus fell considerably after 210 r (values were 57.8% of control). Further irradiation generally increased the SA of the RNA phosphorus which reached maximal values at the 630 r and 2490 r levels (152 and 165% of control values, respectively - Fig. 1). The SA of DNA-P fell likewise at the 210 r irradiation level (62.8% of control). It rose considerably at dose levels 420, 630, and 2490 r (144.5, 164 and 166% of control values, respectively). As in the liver, calculation of RSA values for bone marrow RNA-P and DNA-P showed that the SA changes were not fortuitous, but reflected true changes in the rate of synthesis of these compounds (Fig. 2).

The nucleated cell count per g of bone marrow commenced falling from the onset of irradiation, being in evidence already at the 210 r dose-level. Minimal counts were observed at the cumulative levels of 900 r (30 irradiations) and 1410 r (47 irradiations), when their values reached 39 and 33% of control values, respectively. Further increase of cumulative irradiation dosage actually increased slightly the cell count, but initial control levels were not reached. Bone marrow is known to contain a heterogenous cell population in which the DNA synthesis and cell divisions do not proceed synchronously. Mitosis and DNA synthesis take place only in a small proportion of the cells, within a limited age-group of either the white (myeloblasts, promyelocytes and myelocytes) or red (proerythroblasts, basophilic macroblasts and basophilic erythroblasts) series. The curves presented on Fig. 3 show the changes in total cell population (1), in the absolute number of young cells capable of DNA-synthesis and division (2) and mitotic counts per g bone marrow (3), throughout the period of the present experiment. A comparison of these results clearly suggests that the sharp fall in cell population of the bone marrow took place mainly at the expense of mature cells. The sharp fall in mitoses after 30 irradiations was mainly responsible for the reduction of the cell population in bone marrow at that period. The degree of of cell replacement naturally also depends on an increased number of mitoses.

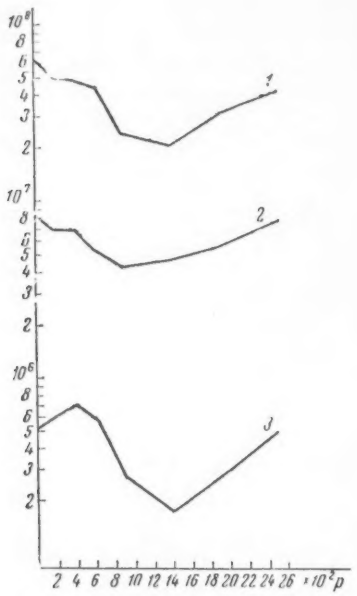

Fig. 3. Total cell counts: 1) Number of cells capable of DNA synthesis and division; 2) and number of cells undergoing mitosis; 3) in bone marrow (per g) after increasing cumulative radiation doses. Abscissa: radiation dose, in r. Ordinates: cell counts (logarithmic scale).

RNA-P per cell was found to be above control levels at most stages of irradiation. At the 210 r dose level the RNA-P content per cell was about twice that of control, after further irradiation the values fell somewhat, but at the cumulative dose of 1410 r the mean RNA-P content per cell was 12×10^{-10} mg compared to $5.3 \times \times 10^{-10}$ in controls. Marked increases in DNA-P content per cell were also observed at the cumulative irradiation levels of 900 and 1410 r (30 and 47 irradiations), when they reached 171 and 183% of control values, respectively.

DISCUSSION

The present study of chronic irradiation once more confirmed the observation that spreading of the irradiation dose over a prolonged period markedly decreased the biological effect [8-12]. This is particularly evident from the survival rates and the results with the bone marrow reported above. As evident from our earlier reports [13, 14] a single irradiation with 1000 r of γ-radiation from Co^{60} caused a mortality rate of 80-90 % among rabbits within 30 days of irradiation. At the time of death the bone marrow of those animals was completely depleted. Within four hours of irradiation DNA synthesis in irradiated rabbits fell below 50% of control values. As shown in our previous reports, after a cumulative dose of 1000 r, delivered at a rate of 50 r per day, the animals survived and regenerative processes in the bone marrow were in evidence, [15]. At that stage nucleic acid levels were 66%, and the SA of NA phosphorus-187% of control values. In those experiments rapid deterioration took place at the cumulative dose levels 1600-1800 r, when the majority of animals died. As a rule the deaths could be ascribed to the failure of hematopoiesis.

In the present experiments, where daily doses of 30 r only were employed, the animals withstood successfully

irradiation doses up to 2490 r (83 exposures), and not one death occurred within 3.5 months.

The analysis of the present results reveals a specific dynamic aspect of the process. This is particularly evident from the consideration of the cell counts and of the NA content and turnover on a single cell basis. The results show that the increased nuclear count per unit weight was not accompanied by complete regeneration in the entire organ. The fall in liver weight and of the liver to total body weight ratio shows the incomplete nature of such regeneration. Thus, despite the absence of any appreciable change of the NA content per g of liver, there were considerable changes in the cell contents of both RNA and DNA. In the bone marrow DNA and RNA content fell appreciably throughout the irradiation period. Since, however, the cell content of NA did not differ from that of control, it must be concluded that the results indicate a depletion of the bone marrow cell population.

Prolonged chronic irradiation caused certain changes in the observed rate of P^{32} incorporation into the NA of both liver and bone marrow. With the irradiation rates and intensities employed, the rate of NA synthesis in the bone marrow rose in a somewhat more steady manner than in the liver, being either above, or within the limits of, control values. DNA is known to be synthesized only during a limited stage of the interphase of the mitotic cycle [16, 17]. It was therefore expected that the increased rate of P^{32} incorporation into the DNA reflected an increased incidence of mitoses. Increased mitotic counts per unit cell population were actually detected in bone marrow at irradiation dose levels of 210, 420, and 630 r, while at cumulative doses of 1420 r mitotic count fell below control levels (Fig. 4). Irradiation with daily doses of 30 r apparently caused intensive cell destruction, which was already apparent after 3, 5, and 7 irradiations. Continued irradiation to a cumulative dose of 420 r did not result in any further fall in total cell population (or in the total number of young cells capable of DNA synthesis); this was possibly due to the increased number of mitoses (Figs. 3 and 4) bringing the cell destruction and regeneration processes to an equilibrium. In the bone marrow of animals which had received 900 r, the total mitotic count fell sharply, but DNA synthesis was quite intensive.

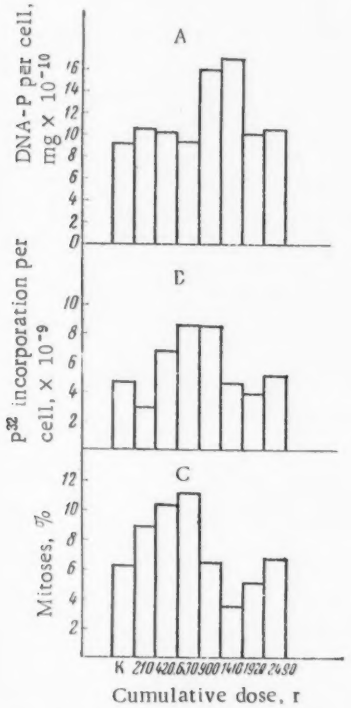

Fig. 4. Calculation of results on the single cell basis. A) DNA-P content per cell; B) Intensity of P^{32} incorporation calculated for a single, young, DNA-synthesizing cell. C) Mitotic counts in per cent of the cell population capable of division.

Expression of P^{32} incorporation into DNA on a basis of a single DNA-synthesizing cell showed that this factor increased steadily after a slight initial fall. While the increase in DNA synthesis was accompanied by a parallel increase in the number of dividing cells, the mean DNA content of a single cell remained constant. A fall in the incidence of mitoses, while the incorporation of P^{32} into DNA continued to rise, was accompanied by an increase of the mean DNA content per nucleus. Thus the disturbance of the normal equilibrium between the DNA synthesis and cell division leads to a formation of cells containing a hyperploid amount of DNA (these results are compared graphically in Fig. 4).

The sharp fall in total cell population after a cumulative dose of 900 r was, apparently, due mostly to cell death, most probably because of failure to enter or to complete mitosis [18, 19]. The situation was similar after a cumulative dose of 1410 r.

Further information about the mechanism, whereby the mean NA content per cell was increased, was afforded by the cytological examination of bone marrow smears. After a cumulative dose of 900 and 1410 r there was an increase in the proportion of young cells of the erythroid series (which are known to be rich in nucleic acids [20]), where hyperploidy is more frequently observed [17] than in the immature forms of granulocytes. The increase in the proportion of erythroid cells is, apparently, due to the raised tendency of the red series towards regeneration, stimulated in the bone marrow by irradiation.

However, it should be noted that further irradiation, with the resultant increase of the cumulative dose, tended to restore the levels of the mean nucleic acid content per cell to near-normal values, with only a slight decrease in erythroblast counts. This suggested the restora-

tion of cell division and the reduction in numbers of cells containing hyperploid amounts of DNA. The results thus suggest that cell division is more sensitive to radiation than the actual process of DNA synthesis, and are in agreement with the published results of Quastler [12, 19], Kelly [21] and a number of others [16, 22].

An increase in the mean DNA content per cell also took place in the liver, where it appeared to be due partially to an increased rate of DNA synthesis (cumulative doses of 420 and 630 r), and later to a low-level continuing DNA synthesis with a simultaneous decrease in the rate of cell division. These results are in agreement with those of Forssberg [23], who observed an increase in the mean DNA content per cell in irradiated ascites, where the number of mitoses, as well as the SA of DNA-P, were below normal levels. These data allow the conclusion that in the liver the processes following irradiation are, at comparable dose levels, similar to those found in the bone marrow. The comparatively long duration of the cell division cycle in the liver determines the low proportion of cell deaths; the main cause of the fall in cell numbers in this tissue is the interference with cell division which, in chronic irradiation, encompasses increasing numbers of cells as the time progresses. The present investigation thus showed that repeated irradiation with 30 r per day caused appreciable changes in both the radiosensitive tissue – bone marrow – and in the relatively radioresistant tissue – liver. Moreover, the depression of DNA and RNA synthesis was more pronounced in the liver. Yet the destructive changes were more pronounced in the bone marrow. This was most probably due to the very high rate of cell division in this tissue.

There still remains the unexplained phenomenon of the increased intensity of nucleic acids turnover during chronic irradiation. A theory on the possible reasons for the increased DNA turnover as a result of the chronic action of deposited plutonium was advanced in one of our previous communications [7]. It is feasible that the same mechanisms are operative in the case of prolonged repeated irradiation. The increase of DNA synthesis may be due to the primary effect of radiation, or of the products of water radiolysis, leading to the breaking of hydrogen bonds which bind two DNA macromolecules [24], or may arise as a result of accumulation and increased concentration of nucleic acid breakdown products [25]. The probability of the latter hypothesis was strengthened by the findings of Fossberg [23] who showed that the restitution of mitotic activity and the increase in the number of dividing cells in ascites following irradiation occurred earlier if the experimental animals were given DNA. The same author also showed that DNA molecules, or their fragments, derived from radiation-killed cells, can pass into growing cells of the ascites population. It thus appears that the intensified rate of cell death, caused by the irradiation rates and intensities employed, could in itself provide an indirect stimulus for the regenerative processes which were observed by both biochemical and cytological means.

Special mention is due to the question of tissue adaptation to high total cumulative radiation doses administered over a prolonged period. There are already indications [11, 12, 19], apart from the present results, that in tissues with a high capacity for proliferation the dialy irradiation may bring about the appearance of new cells with a higher resistance to ionizing radiation, which may affect the probability of adaptation mechanisms in the formation of polyploid cells.

The question of the chemical stability and the biological perfection of the newly synthesized DNA molecules requires more careful consideration, and experimental approaches to this problem are still wrought with considerable difficulties.

SUMMARY

The effect of prolonged irradiation with 30 r per day on the nucleic acids of rabbits' liver and bone marrow has been studied. The fractionation of the irradiation dose over a prolonged period was found to decrease the biological effect.

In the liver the mean RNA and DNA phosphorus content per cell did not vary appreciably; in the bone marrow a considerable fall in nucleic acid concentration was observed almost throughout the entire experimental period.

The mean DNA-P content per cell was markedly increased both in the liver and in bone marrow.

In liver, at cumulative doses of 420 and 2490 r, the rate of P^{32} incorporation into DNA was increased 2.2 and 2.7 times, respectively. The specific activity of the DNA was definitely depressed at cumulative doses ranging from 900 to 1920 r. Changes in the SA of the RNA-P, although not so pronounced, were in the same direction as those in DNA. The specific activities of both the RNA-P and DNA-P in bone marrow were somewhat below control values at the 210 r dose level, and considerably elevated at dose levels 420, 630 and 2490 r.

A theory was advanced about the mechanism of increased rates of DNA synthesis and the changes in mitotic activity following chronic irradiation. It is believed that such a reaction to chronic irradiation confers upon the cells an increased resistance to radiation.

The authors wish to thank L. V. Bogatov and N. A. Korshunikova for the cytological and hematological work in connection with this investigation, and R. N. Zakharova, L. G. Nazarova and V. G. Pestova for technical assistance.

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SOME PROPERTIES OF THE SERINE DEAMINASE OF INTESTINAL AND DYSENTERY BACTERIA

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Chargaff and Sprinson [1] established that the biological catabolism of serine is based on its hydration to α-aminoacrylic acid, which decomposes spontaneously to ammonia and pyruvate. These authors, and also simultaneously Binkley [2, 3], showed that serine deamination by a number of microorganisms actually produced pyruvate.

Although the deamination of serine by various intestinal bacteria has been the subject of special investigations [4, 5], similar work with dysentery bacteria has not, to the author's knowledge, been reported. The present communication adduces evidence to show the ability of dysentery bacteria to deaminate serine, and compares the properties of serine deaminases of intestinal and dysentery bacteria.

METHODS

The microorganisms used in this work were the dysentery strains B. Flexneri 4832 and S. Sonnei 9529, obtained from the Chair of Microbiology, Rostov-on-Don Medical Institute, and a strain of Escherichia coli obtained from the Rostov Institute of Microbiology, Epidemiology, and Hygiene.

Bacterial cultures were grown at 37° for 20 hours, in a medium prepared from dry nutrient agar of the Institute of Microbiology, Epidemiology and Hygiene of the Academy of Medical Sciences of the USSR. The bacterial mass was washed twice with distilled water on the centrifuge in order to remove medium, ground in the cold in a homogenizer with powdered glass and extracted with M/15 phosphate buffer, pH 8.0. The cell-free extract thus obtained was used as an enzyme source. In some experiments the cell-free extracts were prepared by extraction of protoplasts prepared cell lysis with lysozyme*. Cell acetone powder was incubated 30 min at 37° with 1.5-2 mg of lysozyme in M/15 phosphate buffer, pH 7.0. The protoplasts were then removed by centrifugation and the supernatant was used as the enzyme source.

Incubation mixtures (control) contained 1 ml of the bacterial extract, corresponding to 30-40 mg of protein and M/15 phosphate buffer, pH 8.0. DL-serine (10 mg) was added to the first test mixture, while others contained, apart from serine, 4 mg of glucose of ATP. Reactions were allowed to proceed for 2 hours at 37°, and were stopped by the addition of 20% TCA.

Serine deaminase activity was assayed by the appearance of pyruvate, which was determined as the DNP-hydrazone [5], as follows: To the protein-free filtrate of the reaction mixture was added 1 ml of 0.1% solution of 2,4-dinitrophenylhydrazine in 1.2 N HCl, followed by 2 ml of 2.5 N NaOh. The red color of the pyuvate DNP-hyrazone was measured in a FEK-M colorimeter with a green filter.

Color intensities were translated into pyruvate concentrations by the use of a standard pyruvate curve.

RESULTS AND DISCUSSION

Some properties of serine deaminase of E. coli. In experiments with cell-free extracts of E. Coli, incubation mixtures containing DL-serine were found to produce 2.5-6 times the amount of puruvate produced in control mixtures (without serine). The addition of glucose greatly enhanced serine deamination, but ATP was without effect (Table 1).

The results obtained can be better understood in the light of published work on the various pathways of glucose metabolism in some microorganisms. Thus a number of authors [6-8] found that various strains of Pseudomonas catabolized glucose by both the glycolytic pathway and by direct oxidation through 2-ketogluconate, without phosphorylation. It would appear that in the present experiments glucose was catabolized by this latter pathway, while the glycolytic route, with the use of ATP, was absent. Therefore serine deamination in the cell-free E. [illegible] extracts used was taking place at the expense of direct glucose oxidation.

Serine deamination by the cell-free extracts of E. coli was also studied over a range of pH (7.17, 7.38, 7.73 and 8.04). The pH of 8.04 was found to be optimal for this reaction. The activity of serine deaminase was also found to be fully recoverable after acetone treatment.

* The lysozyme preparation was kindly provided by Professor Z. V. Ermolayeva.

TABLE 1. Pyruvate Formation (as Hydrazone) in the Deamination of Serine by Cell-Free Preparations of Escherchia coli (in μg per test).

Control incubation	Test incubations		
	serine	serine + + glucose	serine + + ATP
30	124	474	140
24,3	56	219	52
36	86	272	84
22,7	72,5	261	—
26	84,5	279,5	—
32,5	188,5	442	—

Some of the growth media were reinforced with serine in concentrations 1 mg/ml. Serine deaminase assays on cell-free extracts prepared from bacteria grown on a serine-enriched medium showed that the addition had no effect on the activity levels. Serine deaminase is thus not an adaptive enzyme in E. coli.

In two experiments the acetone preparation of E. coli was treated with lysozyme [9].

Activity assays, as pyruvate formation from serine (in μg) produced the following results:

Control incubations	Test incubations	
	serine	serine + glucose
120	558	642
30	354	408

It follows from the above results that cell-free extracts of lysozyme-lysed E. coli deaminated serine intensively, and that the reaction was enhanced by the presence of glucose.

A comparison of the latter result with that presented in Table 1 tends to suggest that the activity of serine deaminase in lysozyme-lysed E. coli extracts was higher than in extracts prepared by mechanical disruption, although in the former preparation the enhancement of the activity by glucose was less pronounced.

The effect of lysozyme on Gram-negative bacteria is known [10] to consist of the disruption of the cell membrane. In the present experiments the effect of lysozyme was to liberate serine deaminase, which is known to be intracellular enzyme. Furthermore, the specific activity of serine deaminase in the lysed cell extracts, was approximately eight times that in the extracts prepared by mechanical disruption. Thus, in the extracts of lysed cells the protein content of the sample taken for incubation was 20 and 5 mg, respectively, in two separate experiments, while in the extracts of ground cells the protein content of a similar sample was 30-40 mg.

TABLE 2. Pyruvic Acid Formation from Serine (in μg per test) in Cell-Free Extracts of B. dys. Flexneri

Control incubations	Test incubations		
	serine	serine + glucose	serine + ATP
54	240	210	620
52	300	300	767
63	371	371	674
40	288	270	720

The demonstration of serine deaminase in dysentery bacteria. Cell-free extracts of B. dys. Flexneri were found to deaminate serine actively. Thus while in control incubations pyruvate production per test ranged from 40 to 63 μg, in the presence of serine 240 to 371 μg of pyruvate per test was produced. The activity was greatly enhanced by the addition of ATP, but not of glucose (Table 2).

The addition of ATP to the incubation mixtures containing serine caused the synthesis of 620-767 μg pyruvate per test, that is, two to three times the amount formed in the presence of serine alone. This suggests that in Flexner's bacillus glucose catabolism takes place through the glycolytic pathway, with the participation of ATP, and that the energy requirements of serine deaminase are supplied by glycolysis.

The effect of ATP concentration on the intensity of serine deamination by cell-free extracts of Flexner's bacilli was then studied (Fig. 1).

It will be seen from the figure that the addition of 2 mg of ATP to the incubation mixture increased pyruvate production to 720 μg. Further increase of ATP concentration to 3 and 4 mg per test produced a maximal amount of 810 μg pyruvate. Under the present experimental conditions the system was thus saturated with ATP at the level of 3 mg per test.

Serine deaminase assays with cell-free extracts of S. Sonnei produced the following results (expressed as μg pyruvate produced per test incubation):

Control incubations	Test incubations		
	serine	serine + glucose	serine + ATP
48	276	210	312
24	282	270	366
12*	396	378	408

*Lysozyme-lysed cell extracts

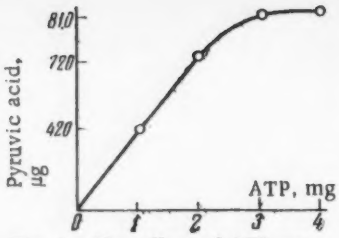

Fig. 1. The effect of ATP concentration on serine deaminase in B. dys. Flexneri.

Extracts of S. Sonnei thus actively deaminated serine. Pyruvate formation in incubation mixtures containing serine was about 11 times that in control mixtures. The addition of glucose to the cell-free extracts did not affect the reaction. ATP had a definite effect, but the stimulation was half as intensive as with the cell-free extracts of B. dys. Flexneri.

SUMMARY

Serine deamination to pyruvate was demonstrated in cell-free extracts of B. dys. Flexneri 4832 and S. Sonnei 9529.

Serine deamination in cell-free extracts of E. coli was stimulated by glucose, while in similar extracts of B. dys. Flexneri and S. Sonnei the reaction was stimulated by ATP.

Lysis of E. coli and of the dysentery bacteria by lysozyme produced extracts with considerably higher specific activities of serine deaminase than did mechanical cell disruption.

Glucose catabolism in E. coli was found to proceed through the direct oxidative pathway, while in the two dysentery bacteria studied the glycolytic pathway operated.

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THE SEPARATION OF 2,4-DINITROPHENYL DERIVATIVES OF SOME AMINO ACIDS BY COUNTER CURRENT DISTRIBUTION

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In spite of the great number of methods worked out in detail for qualitative and quantitative determination of amino acids in mixtures, they cannot always be satisfactorily used.

Thus, the most popular method used at the present time for qualitative determination of amino acids, paper chromatography with development by ninhydrin, isatin, or other reagents, in spite of a number of especially important positive characteristics, has two serious deficiencies. On the one hand, its use does not assure a sufficiently strict identification of the amino acids and their differentiation from each other when the R_f values are close. This is particularly important in studying hydrolyzates of antibiotics, in which we often encounter small distribution of amino acids and sometimes very similar R_f values to those of the usual α-amino acids. On the other hand, the difficulty in preparative separation of the amino acids or their derivatives in paper chromatography makes difficult their exact identification, determination of their configuration by angle of rotation, etc. The most widely used quantitative methods for determining amino acid composition (quantitative paper chromatography, distributive chromatography on starch, ion exchange chromatography, gas-liquid chromatography of amino acid esters, and others) also have deficiencies and are not suitable in all cases. Most of these methods permit separation of only very small amounts of amino acids, which also prevents their use for preparation of the pure substances. Some of the methods mentioned (for example, ion exchange chromatography) cause considerable loss of time and very careful observation of reaction conditions, especially the use of perfectly standard adsorbents.

In connection with our systematic study of the method of counter current distribution we decided to study the conditions of its use for quantitative determination and preparative separation of amino acids or their characteristic derivatives, from mixtures of amino acids, especially from antibiotic hydrolyzates.

As is known, the separation of amino acids by counter current distribution is made difficult by their poor solubility in organic solvents which do not mix with water, and as a rule it requires a large number of transfers. We decided to study chiefly the separation of the 2,4-dinitrophenyl derivatives (DNP) of amino acids. These attracted our attention for a number of reasons. First, in the form of free acids they are well soluble in many organic solvents, and as salts, in water (this makes the choice of a system of solvents much easier, and second, the presence in them of a definite absorption maximum in the ultraviolet at 253 mμ and their yellow color makes their quantitative determination in solution easier. The high optical activity of DNP-amino acids permits carrying out a determination of the configurations of the amino acids; also, the DNP-amino acids are quite stable.

EXPERIMENTAL

Obtaining pure samples of DNP-amino acids. Dinitrophenyl derivatives have been described for almost all the common amino acids [1-6]. They are easily obtained by the action of 2,4-dinitrochlorobenzene on amino acids according to Abderhalden [1] or with 2,4-dinitrofluorobenzene according to Sanger [2]. Since we were not interested in carrying out the reaction under especially mild conditions, we worked with 2-4-dinitrochlorobenzene, carrying on the reaction for eight hours with 5% excess dinitrochlorobenzene. After recrystallization from aqueous acetic acid, most of the DNP-derivatives were obtained in analytically pure form. The constants and analytical data for the products are given in the table. However, recrystallization did not always give a sufficiently pure preparation (for example, in the case of DNP-leucine). To test the purity of DNP-leucine we obtained a curve by the method of counter current distribution (Fig. 1) which showed the insufficient purity of this preparation. By removal of the substance from the tube near the maximum we obtained a preparation with a sufficient degree of purity, as was shown by the curve of counter current distribution given on the same figure.

Selection of phase system. Based on our previous experiments on the partition of isomers of chlorobenzoic

Characteristics of the DNP-Derivatives of Amino Acids

DNP-amino acid	Empirical formula	Melting point		Elementary analysis					
				calculated, %			found, %		
		from literature	our result	C	H	N	C	H	N
DNP-threonine	$C_{10}H_{11}O_7N_3$	178 [3;4] 152 [6]	139—141	42,1	3,9	14,5	42,27	4,03	14,25
DNP-glycine	$C_8H_7O_6N_3$	205 [1] 206 [5] 203—204 [4] 192—193 [3]	192	39,85	2,93	17,5	40,04	3,04	17,55
α-DNP-alanine	$C_9H_9O_6N_3$	178 [1; 5] 172—173 [3]	173—174	42,35	3,55	16,51	42,48	3,72	16,31
α-DNP-amino-butryic acid	$C_{10}H_{11}O_6N_3$	143 [4]	142,5—143,5	44,61	4,15	15,98	44,51	4,19	15,76
DNP-valine	$C_{11}H_{13}O_6N_3$	185 [1; 5] 183 [3] 184 [4]	180—181	46,64	4,62	14,8	47,08	4,82	15,4
DNP-leucine	$C_{12}H_{15}O_6N_3$	131—132 [3] 203 [1] 126 [5]	125—126	48,48	5,08	14,13	48,39	5,19	14,08
DNP-norleucine	$C_{12}H_{15}O_6N_3$		103—110	48,48	5,08	14,13	48,42	5,28	14,03
DNP-phenyl-alanine	$C_{15}H_{13}O_6N_3$	204—206 [3]	205—207	54,38	3,95	12,68	54,22	4,18	12,84

acid [7] we first tested the system butanol-phosphate buffer solutions with varying pH. However, independent of the pH value of the water phase, the DNP-amino acids studied were found in the light phase. The same result was obtained when the buffer solution was replaced by 5% $NaHCO_3$ and even 0.1 N NaOH. Thus it was shown that the use of butanol as the light phase is impossible. Better results were obtained when butyl acetate was used as the light phase and aqueous buffer solutions with different pH values were used as the heavy phase.

In this sytem small changes in pH strongly affected the value of K, and, as would be expected theoretically, there was a linear relation between the pH value and the logarithm of the distribution coefficient (lg K) (Fig. 2). As Fig. 2 shows, there is a great difference between the distribution coefficients of the DNP-derivatives of different amino acids which permits us to continue the attempt to separate them by the method of counter current distribution. For the solution of this problem it was necessary to have direct experiments on the distribution of mixtures with known composition.

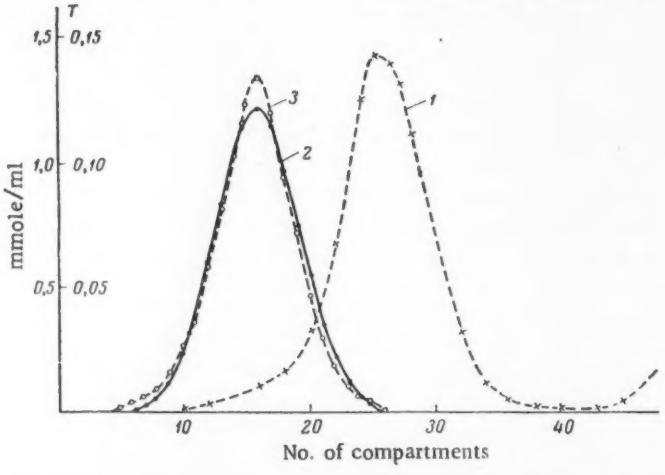

Fig. 1. Distribution curves of DNP-derivative of leucine obtained from the ordinary form of the amino acid (1), and from the amino acid purified by counter current distribution (2). 3) Theoretical curve; scale mmole/ml - for curve 1); T (amount of substances in each compartment) - for curves 2 and 3.

Colorimetric determination of 2,4-dinitrophenyl derivatives of α-amino acids. Even after the choice of the solvent system and determination of the distribution coefficients, it was found that the water and butyl acetate solutions of DNP-amino acids of the same concentration had different extinctions, and, on the other hand, the same extinction corresponded in water and butyl acetate solutions to different concentrations of substances. It was also shown that neither water nor butyl acetate solutions obeyed Beer's Law completely. Therefore, for exact determination of the DNP-amino acid concentration it was necessary to have for each amino acid two calibration curves, and the exact determination of the content of the substance could be obtained only within definite limits of concentration. As an example we give in Fig. 3 (A and B) the calibration curves for butyle acetate and water solutions of some dinitrophenylamino acids.

Using the calibration curves from Fig. 3 (A and B) we can determine from the intensity of color of the butyl acetate and water solutions the content of amino acids with a sufficient degree of accuracy. In practice, however, in most experiments we proceeded somewhat differently for the construction of distribution curves. At the end of the experiment we added to each compartment 0.5-1 ml of 10% H_2SO_4, so that the DNP-amino acid was transferred practically completely into the butyl acetate. This permitted determination of the content in each compartment by colorimetric determination in the light phase only.

Course of the experiments. The experiments on counter current distribution were carried out in a modified apparatus of the firm of Towers and in an apparatus constructed by Avgul, Baikina and Khokhlov [8]. Depending on the particular problem, we carried out from 50 to 225 transfers. The treatment of the results was carried out by the method described previously [7].

RESULTS

We studied the distribution of the dinitrophenyl derivatives of glycine, α-alanine, α-aminobutyric acid and α, γ-diaminobutyric acid, valine, leucine, norleucine and α-phenylalanine, threonine, and others. We found that over a sufficiently wide interval of concentrations all these substances were distributed in accord with the theoretical calculated curves. The experiments on distribution of artificial mixtures of dinitrophenyl derivitives of different amino acids showed that in the case of amino acids differing by more than one methyl group we could carry out a good separation of their DNP-derivatives even with a comparatively small number of transfers. As an example, in Fig. 4 we give the curve of distribution of the ternary mixture of dinitrophenyl derivatives of glycine, α-aminobutyric acid, and leucine, and in Fig. 5, of α-alanine, valine, and norleucine. For the characteristics of the accuracy of the method we can note that in the first case in the distribution of the model mixture of these substances we determined respectively 106, 101, and 104% of the amount introduced. The slight excess over 100% can be explained by the use of techni-

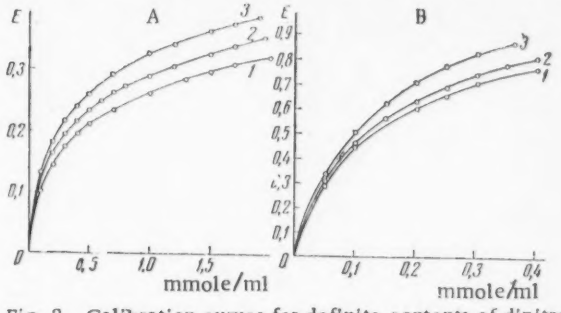

Fig. 3. Calibration curves for definite contents of dinitrophenyl derivatives of some α-amino acids in butyl acetate A) and in 1/15 M phosphate buffers B), pH 5.4. 1) DNP-glycine; 2) α-DNP-alanine; 3) DNP-valine.

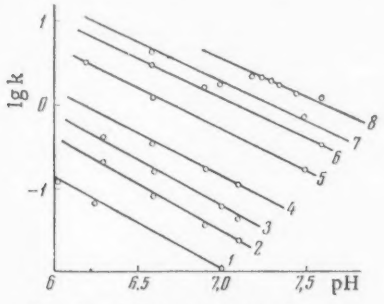

Fig. 2. Relation of lg K of some DNP-amino acids to pH value of the water phase in the system phosphate buffer (1/15 M)-butyl acetate. 1) DNP-threonine; 2) DNP-glycine; 3) α-DNP-alanine; 4) α-DNP-aminobutyric acid; 5) DNP-valine; 6)DNP-leucine; 7) DNP-norleucine; 8) α, γ--di-DNP-aminobutyric acid.

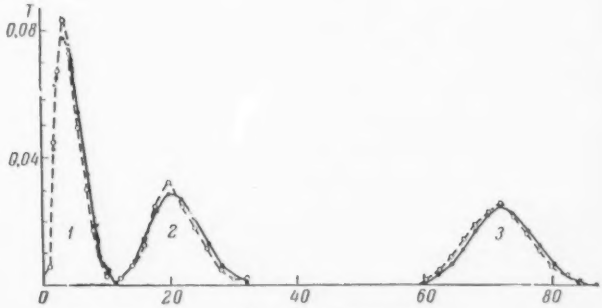

Fig. 4. Distribution of a mixture of 3 dinitrophenyl derivatives of α-amino acids between butyl acetate and 0.2 M phosphate buffer, pH 6.9. T) amount of substance in each compartment; o-o-o theoretical curve; x-x-x experimental curve; 1) DNP-glycine; 2) α-DNP-aminobutyric acid; 3) DNP-leucine.

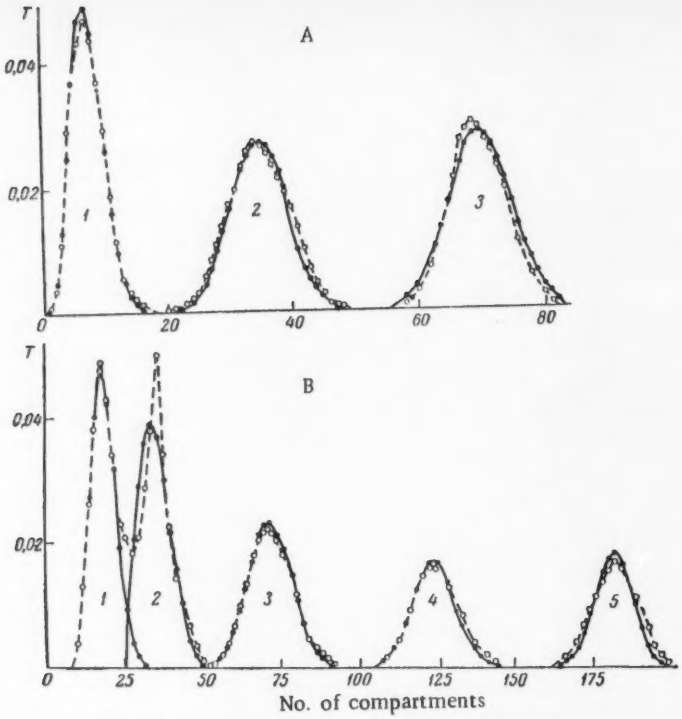

Fig. 5. Distribution of a mixture of DPN derivatives of α-amino acids between butyl acetate and 1/15 M phosphate buffer. A) pH of phosphate buffer 7.0; 1) α-DNP-alanine; 2) DNP-valine; 3) DNP-norleucine; B) pH of phosphate buffer 6.7; 1) DNP-glycine; 2) α--DNP-alanine; 3) DNP-aminobutyric acid; 4) DNP-valine; 5) DNP-leucine.

cal butyl acetate, which yellowed slightly during the experiment.

In work with a large number of transfers (on the order of 200-225) we made a satisfactory separation of a mixture of DNP-derivatives of glycine, α-alanine, α-aminobutyric acid, valine, and leucine (Fig. 5).

SUMMARY

The method of counter current distribution can be used for separation of dinitrophenyl derivatives of α-amino acids, if they are present in comparatively small amount. It permits determination with sufficient accuracy of the ratio of different amino acids and the isolation of the separate dinitrophenyl derivatives.

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THE STRUCTURAL PROTEINS OF SMOOTH MUSCLE OF WARM BLOODED ANIMALS

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In our previous work on the structural proteins of smooth muscle of dog stomach [1-3] we established the presence of an obvious difference between them and the structural proteins of striated muscle of the same animal. This difference was observed in study of the curves of salting out the salt extracts from muscle with solutions of $(NH_4)_2SO_4$ of different concentrations by the method of Derrien-Snellman [4-5].

When we salted out extracts obtained from smooth muscle we found on the precipitation curves two proteins (appearance of two peaks) which did not precipitate from extracts of striated muscle. The first of these proteins had the properties of an actin [1-3], the second was still not identified.

In the literature there is described the presence of an actin peak in $(NH_4)_2SO_4$ salting out of the contractile protein complexes obtained by precipitation with tenfold dilution with water of the salt extract of muscle from the esophagus [6] and muscle from the uterus [5]. We [2] also made an analogous observation on the appearance of an actin peak in salting out by repeated precipitations of the contractile complex of proteins from dog stomach muscle.

In the present work we have set ourselves the problem of establishing some characteristics of the particular salting out curve which we found for the structural proteins of dog stomach also for the structural proteins of smooth muscle (stomach) of warm blooded animals in general. At the same time we wished to explain the presence or absence of observed differences in salting out the smooth muscle proteins in animals belonging to different classes and orders of the warm blooded animals.

In our first work, removal of the structural proteins of muscle was carried out with 0.5 M KCl, without preliminary removal of the globular proteins. This could distort the salting out curves, since the globular proteins and also the protein enzymes found in the group of myogen begin to salt out at concentrations above 30% saturation with $(NH_4)_2SO_4$ [7-10].* The present work should test the size of such an effect and establish its possibility by preliminary removal of globular proteins from the muscle homogenate.

MATERIALS AND METHOD

We studied stomach muscle ground in a meat grinder or cut up by hand (with scissors) from dogs, pigs, rabbits, and cows (all four parts of the stomach separately), pigeons, chickens and man. The number of experiments carried out with each of the samples was respectively 3,4,5,4,2,3,3. The stomachs of dogs, rabbits, pigeons, and chickens were removed immediately after killing the animals in the laboratory. The cow and pig stomachs were obtained from the city slaughter-house from freshly killed animals. The healthy portion of human stomachs was taken from patients suffering from round ulcers of the stomach immediately after resection.

All the manipulations in the treatment were carried out with careful cooling of the material. After removal of foreign tissue (mucous, serous, fatty, etc) and mincing, the muscle homogenate was extracted with 0.05 M KCl with a phosphate buffer, pH 7.0-7.2 to remove most of the globular proteins (5-6 ml of solution per 1 g of tissue). After a day the liquid was filtered through linen, and the mince was treated with five volumes of 0.5 M KCl + 0.01 M KH_2PO_4 + 0.02 M Na_2HPO_4, pH 7.0-7.1. Extraction of the structural proteins of smooth muscle continued for 1-2 days. The salt extract, filtered through linen and freed from chance particles of tissue by centrifuging, was dialyzed against the same buffered solution of 0.5 M KCl to remove the low molecular weight substances, and then was studied.

In a series of test tubes [29-30] was placed 1 ml of protein extract. In the first tube, which served as the control, was poured 6 ml of 0.5 M KCl. In the remaining tubes we poured 6 ml of mixture saturated at room temperature of a solution of $(NH_4)_2SO_4$ and 0.5 M KCl, freshly neutralized to pH 7.0-7.1 by an ammonia solution. The concentration of $(NH_4)_2SO_4$ was progressively raised and included the region from 8 to 70-75% saturation (final concentration). After careful mixing, the tubes stood overnight at room temperature. The precipitate was removed by filtration and the optical density

* This was also reported by V. A. Yur'ev in a discussion of our work [11].

of the filtrate was determined at 278 mμ in an SF-4 spectrophotometer.

The results of the measurements were expressed in the form of a "salting out curve" in which we laid out on the abscissa the salting out concentration of $(NH_4)_2SO_4$, expressed in percent of saturation of the solution (C). On the ordinate was laid out the value of $(-\Delta S/\Delta C)$ where ΔS is the change in extinction with change in concentration of $(NH_4)_2SO_4$ (ΔC). The calculation was carried out for each adjacent pair of experimentally found points. Since the initial samples of extracts in most cases were opalescent, not permitting quantitative determination of the value of light absorption of the proteins, we did not try to express the curve in the form of percent change in extinction with rising concentration of $(NH_4)_2SO_4$, as was done by Snellman and Tenow [5].

In all the experiments we determined the content of protein N in the extracts (modified Conway method). In most of the studies the protein concentration in the solutions for salting out was brought to 0.6–0.9 mg N/ml by the corresponding dilution with 0.5M KCL.

RESULTS AND DISCUSSION

In Fig. 1 we give the separate experiments with salting out the salt extracts of stomach muscle of humans, pigs, pigeons, and rabbits. The forms of the curves have considerable similarity. The number of peaks on them and the distribution of these peaks is much alike.

For comparison of all of our material we have drawn up Table 1 in which for each of our animals we have given the average values for distribution of the peaks, expressed in the form of percent saturation of $(NH_4)_2SO_4$;

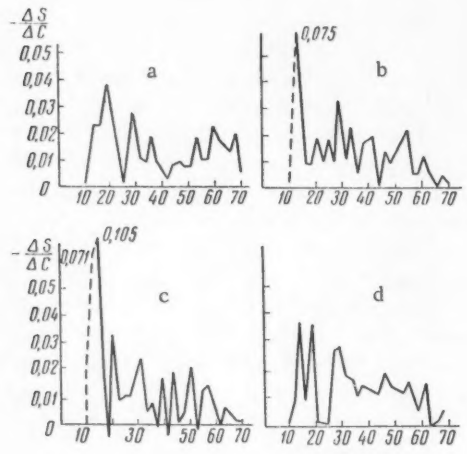

Fig. 1. Salting out curves of structural proteins of stomach muscle. a) Human; b) rabbit; c) pig; d) pigeon. Abscissa-$(NH_4)_2SO_4$ concentration in % of saturation. Ordinate–change in extinction (ΔS) with change in $(NH_4)_2SO_4$ concentration.

average height of each peak, expressed in the form $(-\Delta S/\Delta C)$ and dispersion values for each peak.

When we compare the experiments made on different animals, we find a peak of actin present on the salting out curves of all of them. In some animals in the zone of salting out actin we note the appearance of two peaks: the more constant one, found in all the animals without exception, lies between 12-16% saturation of $(NH_4)_2SO_4$, and the less constant lies between 18-23% saturation. The latter peak in the experiments on birds (pigeon, chicken), where it occurs at 23% saturation of $(NH_4)_2SO_4$, would seem to be due to the salting out of another protein which appears at 25% saturation. However, in chickens, this latter protein gives an independent salting out peak. Therefore we refer the protein which precipitates at 23% saturation to the group of actin-like proteins.

The observed actin peak in the salt extracts of smooth muscle of all of our warm blooded animals indicates that it is a characteristic component of the curves of salting out in smooth muscle in general, and is not a peculiarity of the smooth muscle of dogs. We can also accept this on the basis of the literature data on the contractile protein complexes of smooth muscle of pig esophagus, human uterus, and dog stomach [2,5, 6].

It is impossible to estimate the amount of actin in the smooth muscle extracts solely from the value of optical density, first because of the presence in the protein extracts of cloudiness, which is sometimes considerable (dog, pig, cow). This cloudiness, which disappears when the actin is salted out, simulates a strong absorption at 278 mμ because of light scattering.

The protein salted out in the zone of salting out actin (according to the literature, between 10-20 % saturation of $(NH_4)_2SO_4$ [5]) is certainly not homogeneous. This is indicated by the frequent appearance of a second salting out peak; along with the peak at 12-16% saturation of $(NH_4)_2SO_4$ is a peak at 18-23% saturation. In previous work [2] we noted an abundant inactive protein in the actin preparations isolated from smooth muscle by salting out, which indicates the contamination of actin by other proteins. Whether this is denatured actin or an independent protein of smooth muscle, with similar salting out properties to those of actin is unknown.

There is a peak at 25% saturation of $(NH_4)_2SO_4$ on the salting out curve for humans, dogs, rabbits, cows, and chickens. It would hardly be right to identify it solely by its zone of salting out. In the literature on the proteins of striated muscle there are some still poorly studied "extra proteins" in the corresponding zone of $(NH_4)_2SO_4$ concentrations: the D protein of Amberson and co-workers [12] and the protein isolated by Villafranca [13]. In their properties these proteins have a general resemblance to tropomyosin B [6], but they differ strongly from its zone of salting out (Table 2). They

TABLE 1. Average Distribution (in % Saturation $(NH_4)_2SO_4$. I), Dispersion Value (II), and Height ($\Delta S/\Delta C$) of Peaks (III) of Salted Out Structural Proteins of Stomach Muscle in Different Species of Warm Blooded Animals

Human	*I*	14	19	25	29	35	43	48	54	—	60	66	69
	II	13—14	18—21	25	29	33—37	42—45	48	53—55	—	59—63	66	68—71
	III	0,045	0,032	0,016	0,028	0,015	0,021	0,013	0,013	—	0,020	0,031	0,024
Dog	*I*	14	18	25	29	35	40	48	54	—	62	65	
	II	13—14	18	23—27	29	33—36	39—42	46—50	50—55	—	61—62	65	
	III	0,123	0,023	0,033	0,020	0,014	0,013	0,012	0,009	—	0,010	0,006	
Pig	*I*	13	21	—	29	37	40	45	52	57	62	—	67
	II	12—14	18—23	—	27—31	36—38	40	44—46	51—53	56—58	62—63	—	66—68
	III	0,181	0,028	—	0,038	0,018	0,028	0,022	0,019	0,016	0,007	—	0,006
Rabbit	*I*	14	19	24	30	36	41	47	54	—	60	65	69
	II	12—16	18—21	23—25	29—31	33—38	40—42	46—48	53—55	—	57—61	63—66	68—70
	III	0,063	0,039	0,023	0,023	0,020	0,018	0,014	0,014	—	0,011	0,007	0,009
Cow (rumen)	*I*	12	20	25,5	33	36	40,5	45	—	56	62,5	66	
	II	12	18—21	25—27	32—33	36	40—41	44—46	—	55—58	62—63	66	
	III	0,085	0,032	0,022	0,018	0,019	0,020	0,018	—	0,021	0,012	0,010	
Pigeon	*I*	14	23	28,5	33	37	42	46	55	—	62	66	69
	II	14	23	28—29	33	36—38	42	46	55	—	62	66	68—70
	III	0,037	0,036	0,037	0,028	0,025	0,022	0,010	0,016	—	0,016	0,006	0,006
Chicken	*I*	16	23	25	31	37,5	41	44	49	54	58	62	
	II	15—17	23	25—26	31	37—38	40—42	44	48—50	53—55	57—59	61—63	
	III	0,035	0,033	0,030	0,023	0,018	0,016	0,020	0,019	0,017	0,010	0,011	

TABLE 2. Zone of Salting Out by $(NH_4)_2SO_4$ of Different Structural Proteins of Muscle

Protein	Percent saturation of $(NH_4)_2SO_4$	
	literature data	data of our work
Actin	18 [6], 9—20 [5], 14—20 [1]	12—16
"Extra protein"	23—43 [12], 18—24 [13]	18—23
Tropomyosin A (paramyosin)	33 [14], 28—40 [15]	25—26
Actomyosin	32—33 [17], 32 [6], 28—32 [5], 32 [18], 29—32 [1]	29—33
Myosin	40—43 [19], 35—50 [13], 37—46 [17], 44 [6]	35—37
	33—45 [5], 31—35 [20], 37,5 [18], 37—42 [21], 31 [22]	40—43
L-Meromyosin	33 [18], 32 [21], 32 [22]	—
N-Meromyosin	45—55 [5], 42—45 [23], 42 [21], 45 [22]	45—48
Tropomyosin B	50—66 [19], 50 [24], 45—60 [25], 47—65 [28]	52—56
	50—60 [26], 60—68 [27], 55—64 [5], 48—55 [12]	57—62
Nucleotropomyosin	50—66 [19], 70 [29], 64—70 [5]	65—69
Tsao protein	80 [30]	—

Remarks: The literature sources cited [19, 26, 27] related to a study of the proteins of fish muscle; [5, 14, 15, 17, 24] to the proteins of mollusk muscles; [28, 29] to different vertebrates and invertebrates. In the other sources cited there were studies of muscle proteins from muscles of mammals: striated, cardiac, and smooth.

stand much closer in salting out properties to the tropomyosin A of mollusks which is evidently identical with paramyosin. The presence of paramyosin in vertebrate muscle has not hitherto been described.

Ivanov and co-workers [16] found in myofibrils obtained from smooth muscle a large amount of protein fraction T ("extra protein" obtained by foreign authors), that is, protein not belonging to actin, myosin, or actomyosin. We can assume from these results that the smooth muscle protein salted out at 25% saturation of $(NH_4)_2SO_4$ is one of the components of this fraction.

In the pigeon this peak is shifted to 28% saturation of $(NH_4)_2SO_4$, thus falling in the zone of salting out actomyosin. However, in the pigeon there is a peak at 33% saturation which is evidently actomyosin. Therefore the peak at 28% saturation evidently corresponds to the peak at 25% saturation in the other animals. The size of the peak at 25-28% saturation of $(NH_4)_2SO_4$ is quite large: $-\Delta S/\Delta C$ varies around the value 0.020. If we consider that the protein which precipitates at this $(NH_4)_2SO_4$ saturation can consist to a considerable degree of the D protein of Amberson which has very low specific absorption at 278 mμ [12], then we can speak of a very considerable content in smooth muscle of a protein which precipitates at 25% saturation.

In all the animals studied there was always present a peak of salting out at 29-33% saturation of $(NH_4)_2SO_4$. This peak corresponds to the peak of the actomyosin of striated muscle, as Table 2 shows. Since the salt extract of smooth muscle shows a decreased viscosity after ATP addition which is characteristic for actomyosin [31], we must think that this peak at 29-33% saturation actually corresponds with the precipitation of the actomyosin of smooth muscle. To judge by the height of the peak of salting out, there is considerably less actomyosin in smooth muscle than in striated. The experiments of Ivanov and co-workers, carried out by an entirely different method [16] also indicates this.

From two to three small peaks fall in the zone of salting out myosin, L- and H-meromyosin (35-50% saturation of $(NH_4)_2$, SO_4, Table 2). More exact identification

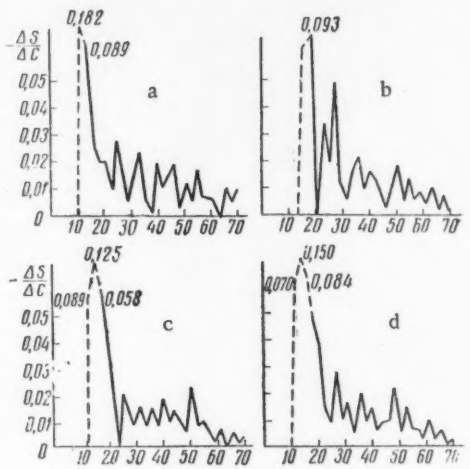

Fig. 2. Salting out curves of structural proteins from the muscle of different portions of cow stomach. a) rumen; b) abomasum; c) reticulum; d) psalterium. Designation of axes the same as in Fig. 1.

TABLE 3. Average Heights of Peaks ($-\Delta S/\Delta C$) in Fractional Salting Out of Extracts (0.5 M KCl) from Dog Stomach Smooth Muscle after Preliminary Extraction by 0.05 M KCl and without It

Extracted material	Salting out concentrations of $(NH_4)_2SO_4$ in % saturation										
	4—16	25	29—30	34—35	37	40—42	47—48	52—54	58	61—62	65
Preliminary extraction with 0.05 M KCl	0,123*	0,033	0,021	0,008	—	0,010	0,010	0,009	—	0,005	0,012
Without preliminary extraction	0,112*	0,033	0,022	0,019	0,028	0,023	0,017	0,018	0,017	—	0,017

*Solution opalescent.

of the separate peaks with definite proteins is impossible at the present time. The same can be said of the peaks in the zone of tropomyosin and nucleotropomyosin (50-75% $(NH_4)_2SO_4$ saturation). The presence in this zone of several peaks indicates that within these limits several individual proteins are salted out.

When we considered the salting out curves for the different sections of the cow stomach (4 experiments), we could not find any notable difference (Fig. 2). Evidently the structural proteins in cow muscle are qualitatively the same. If there are small differences in their quantitative composition, they are not great enough to be determined by our method. We can observe that in parallel studies the most similar salting out curves were found for the psalterium and the reticulum. The curves obtained with extracts from the rumen and the abomasum differed in somewhat greater degree. It is possible that study of more material would lead to a clearer difference between the musculatures of the functionally different division of ruminant stomachs.

Certainly only the accumulation of much more material will permit solution of the question of species differences between the salting out curves of structural proteins from smooth muscle of different warm blooded animals. However, it seems to us that some marked tendencies can be found even now. Among these we should mention the rareness of doubling of the peak of actin in the dog (counting also the experiments published earlier; more than 20 experiments in all); the absence or small size of the salting out peak at 25% $(NH_4)_2SO_4$ saturation in the pig; the shift in distribution of the actomyosin peak to 32-33% saturation (instead of 29% saturation) in cows and pigeons. The fact observed in this work of the constant appearance on the salting out curves of smooth muscle structural proteins in different warm blooded animals of a peak for actin and of proteins which precipitate at 25% $(NH_4)_2SO_4$ saturation, in relation to the small size of the peak for actomyosin gives a characteristic difference in the curves obtained with smooth muscle extracts from the curves given by extracts of striated muscle.

In order to explain the effect on the course of the salting out curves of a preliminary extraction of the muscle tissue with weak KCl (0.05 M), we compared (Table 3) the maximum obtained in the salting out peaks in experiments with preliminary extraction of the tissue and in experiments with muscle extracted directly with 0.5 M KCl. The latter experiment was taken from the work which we published before [1].

As is known, the first three peaks correspond to salting out of actin, unknown protein, and actomyosin and have about the same size in both series of experiments. Evidently globular proteins are not mixed with proteins which precipitate out at $(NH_4)_2SO_4$ concentrations less than 34-35%. Beginning with the peak at 34-35% saturation and in higher concentration of $(NH_4)_2SO_4$ admixtures of precipitating globular proteins are combined with the precipitating structural proteins of the muscle. Judging by the data of Table 3, in dogs the peaks at 34-35% and 37% $(NH_4)_2SO_4$ saturation consist to a considerable degree of globular proteins of the sarcoplasm. Judging by the salting out picture, in the smooth muscle of the dog stomach there is especially little structural protein (not extracted by 0.05 M KCl) which resembles free myosin (salted out at 40-42% $(NH_4)_2SO_4$ saturation).

When the salting out curve of structural proteins is to be studied in all parts, it is necessary first to extract the muscle with solutions of small ionic strength. As to the isolation by direct salting out of actin, actomyosin, and other components of the muscle fibril which precipitate at $(NH_4)_2SO_4$ concentrations less than 34-35% saturation, here the preliminary extraction with weak salt solution (0.05 M KCl) can evidently be omitted. Exactly the same results are obtained in experiments with cow stomach muscle. Corresponding experiments with extracts from the stomach muscle of the other animals were not carried out.

SUMMARY

The salting out curves for the structural proteins of smooth muscle (stomach) of various species of warm blooded animals have great similarity. On the salting out curves of extracts from smooth muscle of all the warm blooded animals studied was a clear peak of actin, absent in curves obtained with extracts of striated muscle. In most of the animals in the zone of salting out of actin

two peaks appeared. For the extracts from smooth muscle the presence of a protein which precipitated at 25% $(NH_4)_2SO_4$ saturation was characteristic. In various species of animals different salting out peaks were shifted relative to each other by several percent of $(NH_4)_2SO_4$ saturation.

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THE REASONS FOR THE ACCUMULATION OF COPROPORPHYRIN IN CULTURES OF THE YEAST Candida guilliermondii

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Recently, various microorganisms have been widely used for study of the path of porphyrin biosynthesis in the living cell [1-3]. In some species of microbes it is easy to produce disturbances of porphyrin metabolism which recall in definite respects the state of "porphyria" in macroorganisms. Such species are fortunate model objects for study of pathologies of porphyrin metabolism.

The state of "porphyria" in yeast organisms was first described by Fischer and co-workers [4]. His experiments showed that various strains of the species Saccharomyces cerevisiae, S. ellipsoideus, and Torula (Mineralhefe) under definite conditions of culturing accumulate isomers of coproporphyrin I in the medium. Disturbance in porphyrin metabolism of the yeasts was connected with vitamin deficiency of the medium. Such a connection was clearly shown, for example, in the work of Stich and Eisgruber [5] who were able to prevent the formation of coproporphyrin I in S. anamensis (one of the strains of S. ellipsoideus) by introducing riboflavin into the medium.

Although, depending on experimental conditions, preparations of autolyzing yeasts can form both isomers of porphyrin [6], the accumulation of coproporphyrin III is not characteristic for the "porphyria" of yeast organisms, and up to now has been found chiefly in bacteria. Recently we [7] found in cultures of the asporogenic yeast Candida guilliermondii an accumulation of a large amount of isomer coproporphyrin III. This species is capable of "oversynthesis" of riboflavin and so the reason for the disturbance in porphyrin metabolism must be different than in S. anamensis. However, in the work cited they were not characterized in detail.

The purpose of the present work was a more detailed study of the nature of the factors which permit the occurrence of "porphyria" in C. guilliermondii.

METHODS

The experiments were carried out with cultures of C. guilliermondii (ATCC-9058) obtained from the collection of the Institute for Microbiology, Academy of Sciences, USSR. For its growth we used a medium with the following composition (in 1 liter): sucrose, 20.0 g; $(NH_4)_2SO_4$, 2.0 g; glycine, 2.26 g; KH_2PO_4, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2.H_2O$, 0.3 g; KI, 0.1 mg; Fe as Mohr salt, 0.125 mg; biotin, 1 μg; Burkholder microelement mixture [8]. For creating an accurate Fe concentration, the medium was first purified with 8-hydroxyquinoline according to Waring and Workman [9]. If necessary the sucrose solution was freed from vitamins with activated charcoal. The yeast was cultured at 30°. Its yield was determined turbidimetrically, and the content of riboflavin in the medium was determined fluorometrically.

Coproporphyrin was extracted from the medium according to Schaeffer [10], from the cells by a mixture of glacial acetic acid-ether (1:2) with later washing with 0.1 M CH_3COONa to pH 6.2 and transfer of the pigment into 0.15 N HCl.

The porphyrin concentration was determined on an SF-4 spectrophotometer. Calculations were carried out by the formula of Rimington and Sveinsson [11]. All the experiments were repeated four times.

RESULTS OF THE INVESTIGATION

Effect of period of incubation. At the beginning of the work we established that under conditions of static culture in the medium with glycine the yeast practically completed growth after 3-4 days of incubation (Table 1). Young, multiplying cells did not contain a marked amount of coproporphyrin, and at the same time already a considerable amount of riboflavin was isolated in the medium.

The intensity of formation of coproporphyrin rose on the 4-7 day of incubation, and on further aging of the culture the content of porphyrin in it changed slightly. Parallelism between the intensity of accumulation of riboflavin and porphyrin in cultures of different ages was not found. On the basis of these results, in all further experiments we determined the coproporphyrin content in seven day old yeast cultures.

Effect of aeration conditions. The yeast was grown in a 100 ml Erlenmeyer flask (with 50 ml of medium

TABLE 1. Synthesis in Coproporphyrin and Riboflavin by Cultures of the Yeast C. guilliermondii of Different Ages

Age of culture, days	Yield of yeast, mg/ml	Concentration in μg/g of cell*	
		coproporphyrin	riboflavin**
2	2,07	4,95	150
4	3,06	111,9	445
7	3,00	222,0	600
10	3,15	291,0	650

*Here and in the following we use the dry weight of the cells.

**We calculated the riboflavin in the medium.

in the flask) in two variant experiments: in one variant the flask was placed in a thermostat (static culture) and in the other, on a horizontal shaking machine (130 oscillations per min). The static culture remained in the thermostat until the end of the experiment. To the aerated culture after 36 hours incubation was added a sterile solution of glycine (10 mg/flask) and some of the flasks were again placed in the shaker. The cells with the medium were transferred from the remaining flasks to presterilized 50, 100, and 500 ml Erlenmeyer flasks which were then placed in the thermostat for further culturing of the yeast which was already in the inactive state. After seven days growth, counted from the day of seeding the medium, all variants of the experiment were analyzed.

The results of these experiments are given in Table 2.

These results show that the greatest amount of coproporphyrin is synthesized only by cells grown in static cultures. Their aeration was harmful, since part of the yeast multiplied on the bottom of the flask, under the layer of liquid. The cells which grew on the surface of the medium, in a film, usually after 3-4 days culturing sank into the liquid and settled to the bottom.

The yeast grown under conditions of constant shaking and aeration of the medium on the shaker, even after the flask had been transferred to the thermostat accumulated little coproporphyrin. The thicker the layer of culture liquid in the flask (lower ratio of volume/surface) the less was the pigment. It is very probable that pigment synthesis was carried out by a small amount of yeast grown after transfer of the culture into the thermostat, and that the "porphyria" of C. guilliermondii thus occurred only under relatively anaerobic conditions. With worsening aeration of the medium, the growth of the yeast also decreased.

The effect of aeration on coproporphyrin synthesis was also clearly shown in the case of growth of the yeast on an agar medium, where accumulation of the pigment was absent.

Effect of source of nutritive nitrogen. In these experiments we studied the effect of source of the nitrogen of nutrition in yeast grown in a thermostat on a medium which contained as its nitrogen source either $(NH_4)_2SO_4$ (4.0 g/liter) alone, or a mixture of $(NH_4)_2SO_4$ (2.0 g/liter) with individual amino acids added in amounts equivalent to the nitrogen of 2.0 g of $(NH_4)_2SO_4$ per liter. From the results of these experiments (Table 3) it is clear that accumulation of coproporphyrin in C. guilliermondii cultures occurs only in the presence of organic nitrogen in the medium.

The synthesis of coproporphyrin is particularly strongly stimulated by glycine, serine, and asparagine; more weakly by threonine; histidine and methionine are ineffective. The effect of other amino acids on the synthesis of coproporphyrin was determined qualitatively by visual observation of the fluorescence of the cells and medium in ultraviolet light. It was shown that glutamic and aspartic acids (the first strongly, the second more weakly) permit formation of coproporphyrin. We did not find a red fluorescence of the cells or medium in the presence of alanine, valine, leucine, isoleucine, arginine, lysine, phenylalanine, or tryptophan.

Synthesis of coproporphyrin was also stimulated by a mixture of organic substances such as malt extract (151 μg porphyrin/g), yeast autolyzate (150 μg/g) and vitamin free casein hydrolyzate (33 μg/g). It is interest-

TABLE 2. Effect of Conditions of Aeration of the Cultures on Growth and Synthesis of Coproporphyrin by the Yeast C. guilliermondii

Conditions of culturing the yeast	Vol. of med./surface of medium	Yield of yeast, mg/ml	Conc. of coproporphyrin, μg/g cells
Full time in thermostat	1, 7	1,86	326,2
36 hrs on shaker, then in thermostat	3, 0	4,96	9,8
The same	1, 7	5,56	8,9
The same	0,43	6,40	7,6
Full time on shaker	—	7,28	1,3

TABLE 3. Effect of Source of Nutritive Nitrogen on Growth and Synthesis of Coproporphyrin by the Yeast C. guilliermondii

Source of nutritive nitrogen	Yield of yeast, mg/ml	Concentration of coproporphyrin, μ g/ g cells
$(NH_4)_2SO_4$	4.28	2.2
$(NH_4)_2SO_4$ + glycine	3.02	172.9
$(NH_4)_2SO_4$ + serine	2.77	63.5
" " + threonine	4.47	10.5
" " + asparagine	3.80	43.1
" " + histidine	4.04	2.4
" " + methionine	1.87	2.0

ing to see that addition of succinic acid to the medium also increased formation of coproporphyrin (63.8 μg/ g). The simultaneous action of succinic acid and glycine was weaker (115.5 μg/g) than the effect of glycine alone (172.9 μ g/ g).

Carrying out an intensive biosynthesis of porphyrin required a high concentration of amino acids in the medium. When the glycine content was decreased to 0.1–0.5 g /liter, formation of coproporphyrin was strongly decreased (to 20-30 μ g/g respectively).

Effect of vitamins. Since the yeast C. guilliermondii is auxotropic with respect to biotin, our basic medium contained this substance. Other vitamins of the B group were added to the medium to get concentrations for thiamine, pantothenic acid, and pyridoxine, 0.5 mg /liter; nicotinic acid, 1.0 mg/liter; inositol, 10 mg/liter. Under these conditions growth and synthesis of coproporphyrin by the yeast C. guilliermondii was changed as follows:

Vitamin added	Yield of yeast mg/ml	Coproporphyrin concentration μ g/g cells
Biotin	2.53	72.5
Biotin + thiamine	2.30	167.3
Biotin + pantothenic acid	2.45	101.4
Biotin + pyridoxine	2.49	73.5
Biotin + nicotinic acid	2.45	104.7
Biotin + inositol	2.47	72.7

It is clear from these data that three vitamins of the B group strengthen the synthesis of coproporphyrin by yeast. These are thiamine, which is the most effective, and also pantothenic acid and nicotinic acid which act with the same intensity. None of the vitamins prevents "porphyria" in the yeast. "Subdeficiencies" of the vitamins do not show any marked effect on the growth of C. guilliermondii.

Effect of iron concentration. In a special series of experiments we measured the iron content in the medium within the limits of those concentrations which had a notable effect on growth and riboflavin synthesis by C. guilliermondii [12, 13]. We determined both the coproporphyrin and riboflavin excreted by the yeast into the medium. The results of this series of experiments were as follows:

Concn. of Fe in medium, μg/ml	Yield of yeast, mg/ml	Concentration, μg/g cell	
		Coproporphyrin	Riboflavin
0.010	0.88	5.2	27,800
0.060	2.24	23.9	4,250
0.125	3.00	222.0	600
0.200	2.86	215.0	480
0.500	2.92	205.0	430

These data indicate the important role of iron in regulating the synthesis of coproporphyrin. Actually, at low concentrations of iron in the medium (0.01-0.06 μ g/ml) when C. guilliermondii carries out the "supersynthesis" of riboflavin with great intensity, the cultures form very little coproporphyrin. Only when the cells are assured sufficient iron (indicated by growth of the biomass) does the yeast syntehsize much coproporphyrin, and then the intensity of riboflavin synthesis is decreased. Thus, there is antagonism between these processes. As we showed earlier, addition to a medium which contains 0.125 μ g Fe/ ml of o-phenanthroline or salts of cobalt also causes a sharp fall in synthesis of coproporphyrin. These compounds strongly stimulate formation of riboflavin by C. guilliermondii [14].

DISCUSSION

Coproporphyrin III is an intermediate product in the process of biosynthesis of heme or substances very similar in structure to its tetracarboxyl precursor [15-17]. Therefore the appearance of coproporphyrin in cultures of heterotropic microorganisms usually is connected with damage to the synthesis of heme enzymes.

Suppression of biosynthesis of iron-containing respiratory enzymes can occur due to lack of iron in the medium, and also, in some aerobic forms, with an insufficient supply of oxygen for the culture [10, 18]. In just these cases occurs formation of large amounts of coproporphyrin III by bacteria [2, 10, 19]. Unfortunately, detailed mechanisms of the occurrence of these forms of "porphyria" by the microbe cells have not been worked out.

As the results of our experiments show, insufficient iron is not the reason for the occurrence of "porphyria" in C. guilliermondiii. A factor which determines the formation of cells capable of the biosynthesis of large amounts of coproporphyrin is insufficient aeration of the medium, especially during the period of multiplication of the yeast. The accumulation of porphyrin begins later in aging the cultures. The amount of coproporphyrin synthesized by the yeast under favorable conditions of the medium was considerable (200-300 μ g/g

of cells) and approached the values found in various bacteria [2, 20].

Since the static cultures of C. guilliermondii consist of cells grown under different conditions of assuring oxygen (films and precipitate), yet all the yeast cultures synthesized coproporphyrin with the same intensity, the "productiveness" of porphyrin biosynthesis (calculated on the biomass) of the cell precipitate should be even higher than was shown by our average results. The results of fluorescent microscopy of the C. guilliermondii cells taken from different parts of the culture confirm these ideas [7].

It is very probable that under conditions of oxygen lack in the medium for C. guilliermondii, the formation of heme enzymes is damaged. Injury to the enzyme systems slows the course of many oxidation-reduction processes, leads to worsened growth of the yeast, and also to the accumulation of one of the intermediate products in the biosynthesis of heme. In the absence of a precursor in the medium accumulation of coproporophyrin occurs on a minimum scale, and "porphyria" is not found.

As is known, the pyrrole rings of porphyrins are formed from δ-aminolevulinic acid, a metabolite which occurs in the succinate-glycine cycle [16]. Substances which stimulate the synthesis of coproporphyrin in C. guilliermondii are either direct participants in the succinate-glycine cycle (glycine, succinic acid) or can easily be transfored into compounds of the cycle (serine and threonine into glycine, aspartic acid into succinic acid, and glutamic acid through α-ketoglutaric acid into succinyl-CoA). Photosynthesizing bacteria in which "porphyria" has been found, also actively transform glycine and α-ketoglutaric acid of the medium into the isomer coproporphyrin III [1]. Although both glycine and succinyl-CoA are needed for the formation of δ-aminolevulinic acid, the biosynthesis of coproporphyrin in C. guilliermondii is activated even by the addition of one of these components or its precursor to the medium. Hence the missing compound must enter from the intracellular "background" of metabolites, and the state of the latter must play an important part in the development of "porphyria." It may be that the stimulating action of some of the B vitamins (thiamine, pantothenic acid, and nicotinic acid) on the coproporphyrin synthesis when glycine is present in the medium comes from the intensification of the yeast carbohydrate metabolism which builds up the metabolite "background" of succinyl-CoA.

It is known from the literature [1,5] that nicotinic acid and thiamine are necessary for the synthesis of porphyrins by a suspension of Rh. spheroides, and pantothenic acid strongly stimulates the formation of coproporphyrin I in S. anamensis.

There is no doubt that the degree of uptake of exogenic metabolites in the porphyrin metabolism of C. guilliermondii should depend on the rapidity with which other processes occur and their utilization in cell metabolism.

Young, intensively growing cells which use the amino acids of the medium for construction of proteins and also, perhaps, in energy metabolism, contain almost no coproporphyrin. As was shown, this substance begins to accumulate only in aging cultures (at 4-7 days growth), which reflects the sharp change in nitrogen metabolism of the cells in which multiplication is decreased.

The degree of utilization by the cells of glycine of the medium for formation of coproporphyrin was very small. According to the data of Table 1 we can calculate that in the formation of 917 μg of coproporphyrin synthesized by the yeast in 1 liter of medium only about 0.9 mg of glycine was used, although at the beginning of the experiment 2.26 g of the amino acid was present.

Another example of the dependence of coproporphyrin synthesis on the occurrence of other processes of use of the amino acid (glycine) in metabolism is given by the experiment with use of different Fe concentrations. When the Fe content of the medium is decreased to 0.06-0.01 μg/ml the yeast C. guilliermondii begins to carry out an intensive "supersynthesis" of riboflavin. Since the construction of a molecule of vitamin B_2 uses glycine, there is "competition" between this process and the coproporphyrin synthesis for this amino acid. Although for many bacteria which do not have the "supersynthesis" of riboflavin "porphyria" occurs at low Fe concentrations, C. guilliermondii under these conditions does not carry out the coproporphyrin synthesis, but evidently first uses the glycine for formation of riboflavin. It is necessary to observe that as the culture ages, vitamin B_2 (like porphyrins) is not used in the yeast metabolism and is excreted into the medium, that, is, becomes an individual final product of nitrogen metabolism of the cell.

SUMMARY

Coproporphyrin III is accumulated in cultures of the yeast Candida guilliermondii after completion of growth of the cells; in 4-7 days of incubation under favorable conditions of the medium there is found 200-300 μg of the porphyrin per g of dry weight of the yeast.

Intensive synthesis of this substance occurs only in cells grown under relatively anaerobic conditions in media which contain precursors of the porphyrin or substance genetically related to it. Thus, the formation of coproporphyrin is stimulated by glycine, succinic acid, serine, threonine, asparagine, aspartic and glutamic acids, and also vitamin free casein hydrolyzate, malt extract, and yeast autolyzate.

In media with glycine the synthesis of coproporphyrin is favored by addition of thiamine, pantothenic acid, and nicotinic acid. There is a relation between

the processes of formation of coproporphyrin and riboflavin. Factors which stimulate the synthesis of vitamin B_2 in C. guilliermondii (low Fe concentration, addition of cobalt or o-phenanthroline to the medium) inhibit the accumulation of coproporphyrin.

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THE USE OF PAPER ELECTROPHORESIS IN CHECKING HOMOGENEITY OF SEED PROTEINS

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The principal methods used at present to follow the separation of proteins in a mixture are solution electrophoresis and ultracentrifugation. Without question these methods yield the most accurate results, considering the present state of refinement of other methods. They require, however, comparatively large amounts of protein solutions. Recently, therefore, the method of paper electrophoresis has come to be widely used, since it does not require complex equipment and a few microliters of solution are sufficient. It is as yet inferior to solution electrophoresis and ultracentrifugation in accuracy and sensitivity, mainly because of adsorption of protein on the paper.

For quite some time paper electrophoresis has been used to check homogeneity of various animal proteins. It has been employed very little with plant proteins, especially seed proteins [1], and the sensitivity of the method for these proteins is completely unknown.

In view of the foregoing, we decided to use paper electrophoresis to follow the separation of pea globulins and to determine the conditions which increase accuracy and sensitivity of the method.

Proteins of pea comprise one of the most thoroughly studied systems of seed proteins [2]. By isoelectric precipitation, Danielsson [3,4] and Kretovich et al. [5] obtained electrophoretically and sedimentationally homogeneous preparations of legumin and vicilin from pea. With this method, however, Johnson et al. [6,7] were unable to free legumin from admixtures of vicilin, even with repeated precipitations. After one fractionation, Joubert [8] also obtained quite impure legumin and vicilin preparations.

In view of the contradictory reports, we concluded that it was necessary to follow through the fractionation of legumin and vicilin.

METHODS

Extraction of legumin and vicilin. Two varieties of pea (Pisum sativum) were used, Capital and Victoria; experimental material was obtained from the 1958 crop of the Kishinev University biological station. Seed coats and embryos were removed and the remaining portion of the seeds finely ground. The meal was defatted at 10-15° with petroleum ether in a cold extraction apparatus. Legumin and vicilin were extracted by Danielsson's method [3]. The defatted meal was extracted overnight at 4° with 10 volumes of 1 M NaCl buffered to pH 7.0, and the extract was centrifuged and filtered through filter paper. This clear salt extract was then freed of albumins by addition of $(NH_4)_2SO_4$ to 70% saturation. The precipitated protein was dissolved in 0.2 M NaCl, pH 7.0, and the solution was dialyzed against distilled water. This operation was performed twice. The globulin precipitate obtained after the second dialysis was dissolved in 0.2 M NaCl, pH 9.2, and the solution dialyzed against 0.2 M NaCl, pH 4.7. The precipitate formed during dialysis, which consisted mainly of legumin (fraction L_1), was freed of vicilin by successive reprecipitations (solution in 0.2 M NaCl, pH 9.2, and dialysis against 0.2 M NaCl, pH 4.7). The purity of the successive legumin fractions (L_2, L_3, etc.) was checked by paper electrophoresis. Purification procedures were continued until electrophoretically homogeneous preparations were obtained.

The supernatant liquid obtained after the first dialysis of the globulin mixture against 0.2 M NaCl, pH 4.7, which contained mainly vicilin (fraction V_1), was dialyzed against distilled water. The vicilin precipitate obtained was purified by successive reprecipitations (solution in 0.2 M NaCl, pH 4.7, and dialysis against distilled water). The purity of successive fractions (V_2 and V_3) was checked by paper electrophroesis.

Dialyses were carried out at 2-4°, the remaining operations at room temperature.

Electrophoresis. As is well known [9-14], many proteins are quite strongly adsorbed by paper, which in a number of cases seriously interferes with the successful application of paper electrophoresis to protein separation. If all the fractions of a given protein mixture migrate in the same direction, some of the most rapidly moving protein will be adsorbed along the migration path and will give increased values for the more slowly moving fractions. If the most rapidly moving fraction is

small in amount, it may go undetected because of complete adsorption along the migration path.

To obtain the most reliable results, therefore, it is desirable to keep adsorption to a minimum; this can be done by shortening the migration path.

In the majority of paper electrophoresis devices in use at the present time, evaporation of water from the paper takes place, which gives rise to the flow of liquid. This flow substantially affects both the direction and rate of migration of protein molecules. Thanks to it, the various protein fractions reach characteristic equilibrium positions which depend not on their original position but only on electrophoretic mobility [13, 15].

If it is necessary to determine the content of any one component more accurately, this is most likely to be accomplished by applying the mixture to the paper at a position between the equilibrium positions of this component and its neighboring components. These fractions will then move in opposite directions. If it is necessary to determine the amount of a contaminating protein, the solution must also be applied at a place situated between the equilibrium positions of the main component and the contaminating component; in order to prevent appreciable error, the level of adsorption of the main component at the site of application should not go beyond the limits of the Gaussian curve of this component (Fig. 1).

Correct selection of the application site is therefore of the greatest importance in obtaining the most accurate results possible. In the study of any protein mixture it is necessary to first determine the equilibrium positions of the component proteins and then to select the site of application in accordance with the particular objective of the study.

Electrophoretic separations were carried out in a device of the horizontal type produced in the experimental design shops of the A. A. Bogomol'tsa Kiev Institute of Physiology (EMIB), and also in a horizontal-vertical device, which has been described by Gurvich [16]. In the first experiments it was found that at 4° only the original salt extract of pea meal was amenable to electrophoretic separation. Even after the first purification by precipitation with ammonium sulfate, the proteins could not be separated in the cold because of a marked reduction in solubility at low temperatures. At room temperature, separation was somewhat poorer in the Gurvich chamber than in the EMIB chamber.

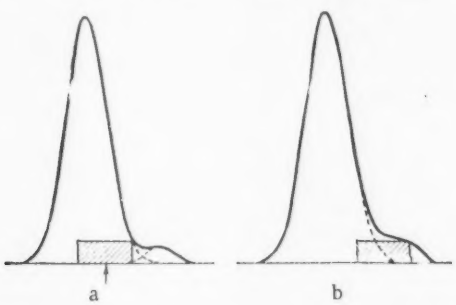

Fig. 1. Diagram of solution application. Application site indicated by arrows. Cross hatched areas correspond to adsorption of the main component at the application site. a) Correct; b) incorrect.

Conditions employed in experiments in the Gurvich chamber were as follows: potential gradient G = 3.7 ± ± 0.1 or 5.1 ± 0.2 v/cm; time t = 6-22 hours at T = = 3 ± 1° or 19 ± 1°; current applied at beginning of experiment 1.4-3.3 milliamps at end of experiment 2.0--5.9 milliamps.

With the EMIB chamber, the best results were obtained at G = 4.7 v/cm, room temperature, and t not less than 6 hours; current applied at beginning of experiment, 2.5-2.8 milliamps, at end of experiment, 3.7-4.7 milliamps.

All experiments were carried out in borate buffer, pH 7.8 and μ 0.25, on Leningrad No. 4 chromatographic paper. Electrophoretograms were 4 cm wide. To prevent edge effects [17], two strips of filter paper about 1.5 cm wide were placed along the sides of the bridge of the EMIB chamber. Electrophoretograms were prepared and treated for the most part according to a method described previously [9], with the following alterations: Papers were dried 10 minutes at 120-130° and treated with a brom phenol blue solution for 10 minutes, after which excess stain was washed off 4-5 time for periods of 2 minutes each. The protein mixture was applied to the paper at such a place that vicilin and legumin would migrate in opposite directions. In the Gurvich chamber, the solution was applied 7 mm from the center on the side toward the anode, and in the EMIB chamber, 2 mm from the center on the side toward the cathode.

The electrophoretograms were quantitatively assessed by elution [9]. It was assumed that legumin and vicilin bind equal amounts of bromphenol blue per mole protein, since they have approximately the same number of basic amino acids [18]. Since 0.01 N NaOH did not extract brom phenol blue from the paper completely at room temperature, elutions were carried out at 50° for 20 minutes. Under these conditions, the extinction of the solution remains practically constant for at least 30 minutes. The electrophoretogram was cut into strips 2 mm wide, from which the bromphenol blue was extracted with 2.5 ml NaOH. At the beginning of the study, extinction of solutions was measured in an FEK-M photoelectrocolorimeter with a green light filter, and later in an FEK-N-57 colorimeter with a No. 6 filter, which gives a 2.25-fold increase in extinction. From the extinction values obtained, a curve was constructed and the percentage composition of the mixtures computed [9]. Where necessary, correction was made for adsorption.

To determine the extent of adsorption of legumin and vicilin, solutions of L_6 and V_3 were applied to sepa-

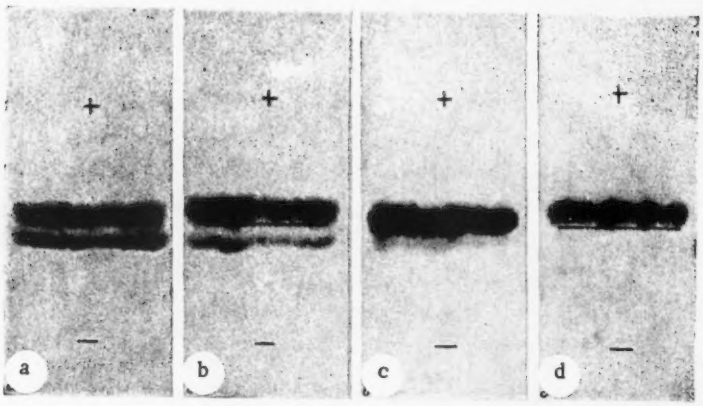

Fig. 2. Electrophoretograms of original mixture a) and of legumin at various stages of fractionation. b) Fraction L_2; c) fraction L_4; d) fraction L_6. Electrophoretogram a obtained in a Gurvich chamber, the rest in an EMIB chamber.

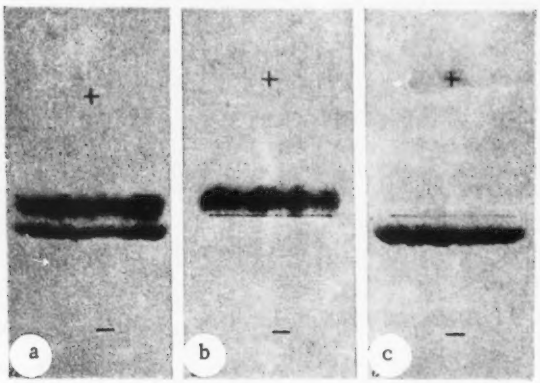

Fig. 3. Electrophoretograms of original mixture a) Final legumin fractions; b) and vicilin; c) Electrophoretograms (a) obtained in a Gurvich chamber, the rest in an EMIB chamber.

rate papers 8.5 cm from the equilibrium positions of the respective proteins, and electrophoresis was carried out under ordinary conditions for 15 hours. The amount of protein in adsorbed "traces" was determined by elution.

RESULTS AND DISCUSSION

The solution fractionated contained 45.0% legumin and 55.0% vicilin. Electrophoretograms illustrating the composition of certain intermediate and final fractions are presented in Figs. 2 and 3.

A characteristic feature of the electrophoretic behavior of legumin and vicilin is that the legumin zone is always more diffuse than the vicilin zone; judging by their diffusion coefficients, we should have found the reverse to be true. Only the electrophoretograms of proteins from Victoria peas were quantitatively evaluated.

The data summarized in the table show that in most cases the accuracy of evaluation of legumin fractions is fully satisfactory. Only in the case of fraction L_2 is the scattering of data large, and the values obtained for this fraction are obviously unreliable. The accuracy of determination of vicilin fractions was markedly lower. Although the relative error for the vicilin component did not exceed 6%, for component x in fractions V_2 and V_3 it was too high (~35%).

For fractions L_5 and L_6 we found that when the solution was correctly applied symmetrical extinction curves were obtained (Fig. 4c), indicating the absence of vicilin. When the solution was incorrectly applied (when it is necessary to correct for legumin adsorption), the vicilin contaminant amounted to 2.8 ± 0.8% of the total. However, this value cannot be regarded as reliable because of uncertainties in the calculation of vicilin content introduced by the association with it of strongly adsorbed legumin. (As we established, under our conditions legumin

Composition of Fractions Obtained during Separation of Legumin and Vicilin Average values of 3-7 Determinations

Fractions	Content, %			σ*
	legumin	vicilin	component x	
L_1	75,1	24,9	—	0,6
L_2	77,9	22,1	—	7,9
L_3	90,0	10,0	—	0,7
L_4	96,2	3,8	—	0,5
L_5	100(?)	0(?)	—	—
L_6	100(?)	0(?)	—	—
B_1	—	83,7	16,3	2,4
B_2	—	86,6	13,4	4,9
B_3	—	89,2	10,8	3,7

*Standard error of determination.

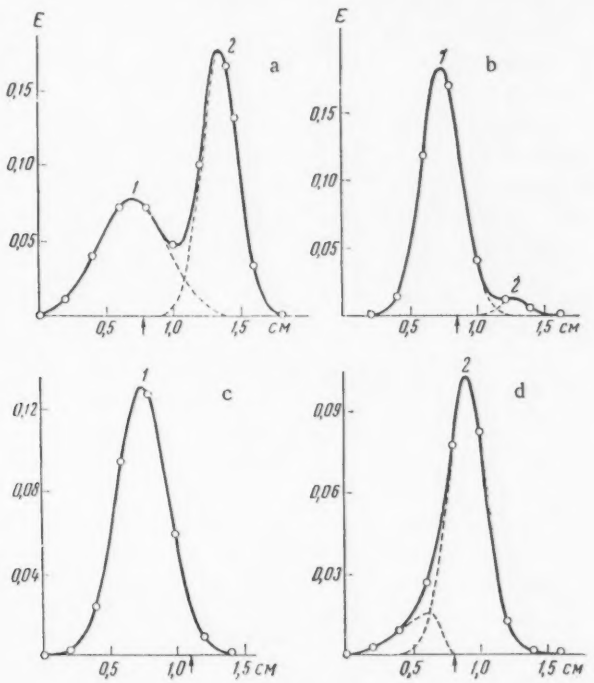

Fig. 4. Extinction curves of certain fractions. a) Original mixture; b) fraction L_4 (legumin adsorption corresponding to ~0.02 units); c, d) fractions L_6 and V_3. 1) legumin; 2) vicilin.

is adsorbed six times more strongly than vicilin.) On the other hand, an impurity of the order of 1% cannot be detected with certainty even with correct application. It is only with a fair amount of justification, therefore, that we can say that the vicilin content of fractions L_5 and L_6 is below 3.5%.

Thus, this method of electrophoresis enables us to detect with a fair degree of reliability vicilin impurities in a legumin preparation of the order of 4%.

For comparison we shall point out that White et al. [19], in a study by paper electrophoresis of human A- and S-hemoglobins whose relative mobilities are close to those of legumin and vicilin, concluded that in the separation of these two compounds the minimum detectable amount of an S-hemoglobin impurity in an A-hemoglobin preparation is ~10%. However, they did not take into account the error introduced by adsorption even though, judging by their photographs in Fig. 1, these hemoglobins are adsorbed to an appreciable extent by paper, and their migration path is quite long (~6 cm).

Our results show that with proper choice of the application site, the minimum detectable amount of admixtures may be reduced at least two-fold. To find these admixtures, however, it is necessary to apply the solution to the chromatogram very accurately.

If it is necessary to detect small admixtures of legumin in vicilin, it is not of such great importance to apply the solution accurately, since the degree of vicilin adsorption is much less than that of legumin adsorption; the error introduced by adsorbed vicilin may therefore be assessed quite accurately. Even in this case, however, it is obviously necessary to apply the solution between the equilibrium positions of legumin and vicilin.

Quantitative analysis of the vicilin electrophoretograms yielded unreliable results. At all purification stages, there was a component migrating in the same direction as legumin but not associated with a separate peak on the curve (component x in Fig. 4d and the table). The amount of this component x fell very slowly with vicilin purification.

On the basis of these data we cannot state to what protein or proteins this component corresponds. It may be composed in part of legumin which failed to reach its equilibrium position, since the latter is not present in amounts sufficient to forestall adsorption. Evidently component x represents to a considerable extent protein possessing a mobility intermediate between that of legumin and vicilin. Perhaps this component in some measure represents associated vicilin, since our conditions favor association to a definite extent [8]. Although it has been found [6, 20] that associated proteins do not differ from monomer molecules in electrophoretic mobility and are therefore not separated from these by solution electrophoresis, we are inclined to think that they can be separated by paper electrophoresis. The basis of this assumption is that probably the association product differs from the monomer in degree of adsorption on paper, and also that these entitites differ in size. It is also possible that component x consists to a definite extent of partially denatured but still soluble vicilin.

In view of our finding that the impurity in vicilin fractions V_1–V_3 is hardly legumin, we decided to limit purification to stage V_3.

SUMMARY

Paper electrophoresis was utilized in the study of pea legumin and vicilin. Legumin is adsorbed by paper much more than vicilin. To reduce error introduced by protein adsorption on paper, the protein mixture should be applied as near as possible to the equilibrium position of the protein studied, so that this latter migrates in one direction, and the other components in the opposite direction. By these means, an ~4% admixture of vicilin in legumin may be detected.

Preparations of legumin and vicilin have been isolated by isoelectric precipitation. After 5 reprecipitations, the legumin preparation contained less than 3.5% of vicilin. The vicilin preparation consisted of two components differing slightly in electrophoretic mobility. The content of the main component was ~90%. The nature of the second component is obscure.

In conclusion, we wish to express our deep appreciation to Professor V. G. Klimenko for the original suggestion and for direction of this study.

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THE ROLE OF NONPHOSPHORYLATING OXIDATION IN TEMPERATURE REGULATION

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Biological oxidation reactions may be divided into two groups according to accumulation of energy in chemical bonds: those coupled to the formation of high-energy phosphate bonds (phosphorylating oxidation-PO), and those not so coupled (nonphosphorylating oxidation-NO).

The functional roles of these two types of reaction have been studied to different extents; PO, which is coupled to ATP synthesis, has been studied quite thoroughly, while the physiological significance of NO remains in many ways obscure. This has given rise to the belief that NO is an ineffective process "unharnessed" to cellular activities, which is characteristic not so much of a normal physiological condition but of a pathological lesion in metabolism (thyreotoxicosis, K- and E-avitaminosis, fever induced by dinitrophenol [1-4]).

The opposite view is that NO is a physiological form of tissue respiration which participates with PO in the metabolism of a normal organism. This possibility is discussed in a number of reviews of oxidative phosphorylation [5-14]; the "physiological validity" of NO has been pointed out in studies of temperature regulation [15-20]. Neifach [9, 16] has formulated a hypothesis of the "physiological effectiveness" of NO as a mechanism of heat production. If this is so, then NO should be maximally activated during abrupt cooling of a warm blooded animal and should assume a decisive role in survival where the effect of cold is quite severe.

This study is devoted to the experimental demonstration of NO activation during abrupt cooling.

METHODS

Experiments were performed on pigeons (Columba livia). The birds were cooled in a refrigerator at -15°. To enhance the effect of cooling, they were sheared. Oxygen consumption was measured according to the method of Kalabukhov [21] at 24° for the control group and at 0° for the cooled group, this measurement being made immediately after cooling.

Body temperature was measured with a rectal thermometer. Coupling of oxidation and phosphorylation was measured in mitochondria isolated from the pectoral muscle, with succinic acid and pyruvic plus malic acid as substrates.

Measurement of oxidation and phosphorylation in muscle mitochondria. The pigeons were decapitated and the pectoral muscle rapidly removed, immersed in liquid at 0° for homogenization (see below), and weighed. The muscle was coarsely minced with knives and homogenized with 5 volumes of a solution containing 0.1 M KCl, 0.05 M tris, pH 7.4, and 0.001 M $MgCl_2$. The homogenizer consisted of a teflon plunger and a glass tube. Nuclei and cell membranes were removed from the homogenate by centrifugation for 10 min at 600 g, and mitochondria were then sedimented by centrifugation for 10 min at 9000 g. All operations were performed at 0-2°. The mitochondria were suspended in the same solution and incubated in Warburg vessels for 40 min at 26° in air. The incubation mixture (2 ml) contained mitochondria (5 mg protein), 40 μM KH_2PO_4, pH 7.4, 200 μM KCl, 10 μM $MgCl_2$, 3 μM AMP, pH 7.4, 150 μM glucose, three times as much hexokinase as that required for the maximum rate of phosphorylation, and substrate (40 μM succinic acid or 40 μM pyruvic plus 4 μM malic acid).

The hexokinase preparation was obtained according to Berger, et al. [22] (5 stages) or according to Sols, et al. [23]. The ΔO_2 was determined manometrically; ΔP was determined by the decrease in inorganic phosphate (P_{inorg}) as described previously [24]. Mitochondrial swelling was measured in an electronephelometer according to change in optical density.

RESULTS

Changes in body temperature during cooling. Within 10-20 minutes after exposure to a -15° temperature, sheared pigeons exhibited a progressive hypothermy (Fig. 1, curve A, 1) if prior to the experiment they had been exposed to warmth for a longer period ("summer" pigeons, which had been kept in a coop heated to about + 15° after the advent of cold weather). Pigeons taken in winter from an unheated coop maintained a body temperature of ~40° for several hours after exposure to -15° (Fig. 1, curve C, 1). It proved possible to develop

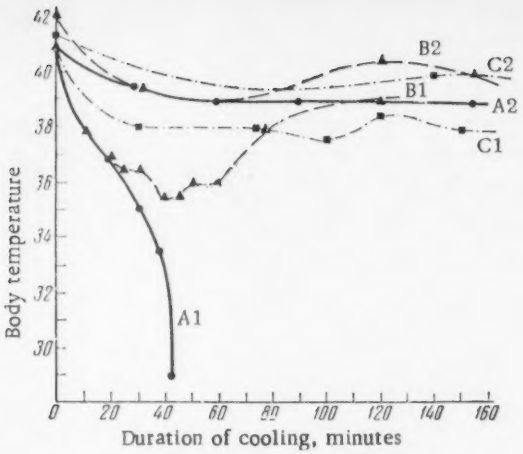

Fig. 1. Changes in body temperature of pigeons during cooling. A, 1) Pigeon kept 4 months at room temperature, first cooling; A, 2) same bird, second cooling; B, 1) pigeon kept 4 months at room temperature and 2 days just before the experiment at 0°, first cooling; B, 2) same bird, second cooling; C, 1) pigeon kept from October through January in an unheated coop, first cooling, C, 2) same bird, second cooling.

artificially the capacity for maintenance of body temperature in the cold in birds which had been kept in warm conditions. This was accomplished by cooling sheared birds until their body temperature had fallen to 5-10°, and then returning them to a warm environment. If these birds are cooled again 1-2 days later, they are able to maintain a body temperature of ~40° for hours (Fig. 1, curve A, 2). An unsheared pigeon kept at 0° for a few days also acquired the capacity to maintain body temperature in the first cooling period, but not to as great a degree as during the second cooling (Fig. 1, curves B, 1 and B, 2).

Stages of adaptation to cold. Investigation of body temperature, oxygen consumption (gas exchange) in vivo, and coupling of oxidation and phosphorylation in vitro* afforded a basis for distinguishing three stages of adaptation to abruptly reduced temperatures.

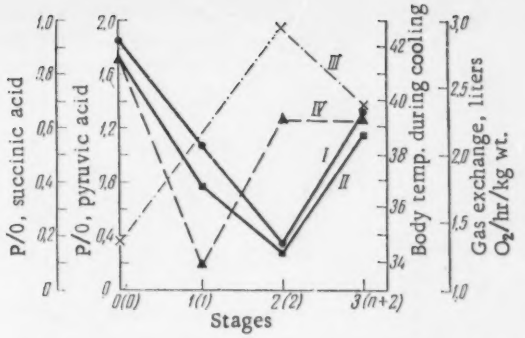

Fig. 2. Changes in P/O, gas exchange, and body temperature during cooling at various stages of adaptation to cold. I) P/O for pyruvic acid; II) P/O for succinic acid; III) gas exchange; IV) body temperature; numbers in parentheses on abscissa – amount of conditioning to cold (n + 2– corresponds to natural conditioning and two artificial conditionings).

Stage I. Some uncoupling of oxidation and phosphorylation (reduction in P/O) during the first cooling of birds which had been kept under warm conditions. This stage is accompanied by progressive hypothermy and a temporary stimulation of gas exchange which is replaced by a decline with fall in body temperature to 8-10°.

Stage II. Further uncoupling during the second cooling 1-2 days after the first cooling. In this stage both body temperature (39-40°) and gas exchange (200-250% of normal) are stabilized.

Stage III. Decrease in uncoupling and in gas exchange. As in stage II, body temperature is near normal. Stage III appears during the second cooling in birds which had previously been adapted to cold by containment in a cold coop ("natural" conditioning to cold). Already in the first cooling period such birds exhibit changes characteristic of stage II in the adaptation of "summer" pigeons (uncoupling, maintenance of normal body temperature, and stable rise in gas exchange). This is strikingly borne out in a comparison of "natural" and "artificial" conditioning to cold:

Adaptation to cold	P/O	Gas exchange, liters O_2/kg wt/hr	Body temperature in the cold
Without cooling	0.85 ± 0.10	1.36 ± 0.035	42°
Artificial	0.15 ± 0.05	2.94 ± 0.135	39.2 ± 0.3
Natural	0.28 ± 0.07	2.76 ± 0.06	38.1 ± 0.4

Note: Measurement of oxidative phosphorylation was made with succinic acid as a substrate.

*E. E. Benediktov, G. M. Maslova, and V. G. Shestakov participated in these experiments.

TABLE 1. Restoration of Pyruvic Oxidase Activity by DPN and Restoration by ATP of Coupling in Mitochondria from Pigeons Adapted to Cold

Exptl. cond.	Amount added, μ moles		Succinic acid		Pyruvic + malic acid		$\Delta O_{pyr}/\Delta O_{succ}$
	DNP	ATP	ΔO	P/O	ΔO	P/O	
Without cooling	—	—	7,05	1,1	4,75	2,05	0,67
Adaptation to cold	—	—	6,90	0,47	3,15	1,02	0,46
after cooling	4	—	—	—	5,40	1,11	0,78
	2	—	—	—	5,25	1,11	0,76
	2	2	—	—	5,10	1,46	0,74

Note. In this and following tables ΔO and ΔP are expressed in μ atoms.

TABLE 2. Restoration by ATP and EDTA of Phosphorylative Coupling in Mitochondria from Pigeons which Had Been Cooled Twice

Exptl. cond.	ATP and EDTA, 2 μmoles each	Succinic acid			Pyruvic + malic acid		
		ΔO	ΔP	P/O	ΔO	ΔP	P/O
Without cooling	—	7,9	8,25	1,04	5,45	11,3	2,07
	+	8,1	11,0	1,36	6,75	15,4	2,28
After cooling	—	6,4	1,4	0,22	2,30	2,3	1,00
	+	6,5	5,1	0,79	3,70	6,5	1,75

In Fig. 2 are shown changes with stage of adaptation in average P/O ratios, body temperature, and gas exchange at low temperature. Analysis of individual variations at the various stages indicates that as adaptation proceeds the range of response of the organism to cold decreases. (This was postulated by Potter [18]). Specifically, it was found that the variation in P/O was greatest during the first cooling; here the full range of responses from unchanged P/O to complete uncoupling was found. During the second cooling P/O values were limited to a range from 0 to 50% of the normal. At the third stage of adaptation, the range of variation was still more contracted: ratios were always reduced, but this reduction sometimes was less than 50% of the original value. This pattern is reflected in the pattern of the coefficient of variation (equal to $\sigma \cdot 100/P$; 0, where σ –standard deviation), which was calculated for the controls of stages I and III (substrate–pyruvic and malic acid):

Stage of adaptation to cold	P/O	σ	Coefficient of variation, %
0	1.82	0.31	17
1	1.06	0.47	44.5
3	1.35	0.28	21

The coefficient of variation for the second stage is not given since here the absolute value of P/O is almost six times lower than the control value, and an increase in the coefficient of variation could have been due to the increased relative error of measurement.

Characteristics of thermoregulatory uncoupling of oxidation and phosphorylation. This study has shown that a change in P/O is strictly correlated with a change in the structural condition of the mitochondria. As Fig. 3 shows, a reduction of P/O at stages I and II is parallel with the decline in optical density of the mitochondria, i.e., with their swelling, while the increase in P/O at stage III is associated with an increase in optical density. Similar changes were noted in the rate of hydrogen transfer at the pyridine nucleotide stage. It is well known

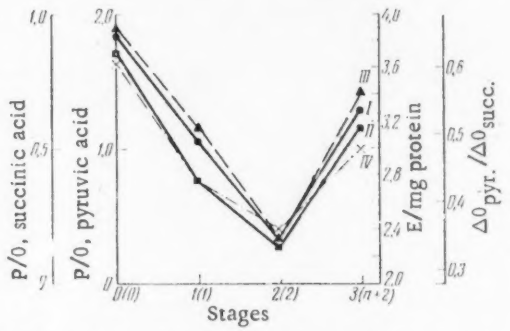

Fig. 3. Changes in P/O, optical density of mitochondria, and ratio of rates of oxidation of pyruvic and succinic acids at various stages of adaptation. I) P/O for pyruvic acid; II) P/O for succinic acid; III) optical density; IV) $\Delta O_{pyr}/\Delta O_{succ}$. Figures on abscissa as in Fig. 2.

TABLE 3. Manifestation of "Coupling" Effect of ATP and EDTA During Isolation and Incubation of Mitochondria from a Pigeon Adapted to Cold

Added to isolation medium		Added to incubation medium		ΔO	ΔP	P/O
ATP	EDTA	ATP	EDTA			
–	–	–	–	5,00	2,10	0,42
+	+	–	–	5,00	2,10	0,42
–	–	+	+	5,55	6,00	1,08
–	–	+	–	5,90	5,60	0,95
–	–	–	+	5,15	2,75	0,54

that inhibition of oxidation through DPN is one of the most sensitive indicators of structural degradation in mitochondria, since pyridine nucleotides pass out of the mitochondria extremely easily during swelling [25]. As a measure of the rate of oxidation through DPN, we selected the ratio of O_2 absorption during oxidation of pyruvic and malic acids, which involves DPN, to that during oxidation of succinic acid, which does not involve pyridine nucleotides ($\Delta O_{pyr}/\Delta O_{succ}$). The parallelism of this ratio and the P/O ratio are evident in Fig. 3. Addition of DPN to an incubation mixture containing mitochondria from pigeons which have been subjected to cooling completely restores $\Delta O_{pyr}/\Delta O_{succ}$ (Table 1).

This treatment has essentially no effect on P/O. This can be partially restored by addition of ATP (Table 1) or, still better, ATP plus EDTA. These compounds increase somewhat the P/O of mitochondria from pigeons which have not been cooled, but their "coupling" effect is particularly strong in mitochondria of birds which have been cooled twice, and in which oxidation is to a considerable degree of the non-phosphorylating type (Table 2).

Thus, use of "coupling" agents reduces differences between control and cooled groups with respect to P/O. In studying the stages of adaptation to cold, we therefore refrained from using PO activators such as ATP and EDTA in the incubation mixture and high concentrations of magnesium in the isolation medium. Under our conditions, P/O of the control was somewhat reduced, but the effect of cooling was manifested most clearly. It is of interest that the "coupling" effect of ATP and EDTA (in concentrations of the order of 0.001 M) shows up only when these compounds are present during incubation of the mitochondria. Isolation of mitochondria in their presence is substantially without effect on the extent of coupling. Evidently temperature is critical in this case: Isolation is carried out at ~0°, and incubation at 26° (Table 3).

A study of mitochondrial swelling showed that optical density at 0° is stable and practically the same in the presence and absence of ATP and EDTA. At 20°, the optical density is affected by ATP and EDTA. When they are present, the mitochondria shrink (optical density increases), and when they are absent the mitochondria swell rapidly (Fig. 4). The study of shrinking and swelling of mitochondria from the pectoral muscle of pigeon will be described in a separate communication.

DISCUSSION

Thermoregulatory coupling as evidence for a functional role for nonphosphorylating oxidation. The rapid and marked activation of NO described above is possible only if there is present in the mitochondria a vigorous NO system and a fully developed physiological mechanism for shifting oxidative pathways. Activation of the NO system is critical under rapid cooling conditions, when unconditioned animals are unable to uncouple respiration and phosphorylation to a sufficient degree, while conditioned ones can do so and survive. Under such conditions, therefore, NO, which is often designated as "ineffective," proves to be more effective physiologically than PO.

The following considerations attest to the fact that NO is one of the natural mechanisms for emergency mobilization of heat.

1. The maximum activation of NO coincides with emergence of the capacity to maintain normal body temperature during abrupt cooling (second cooling of pigeons which had been kept at warm temperatures).

2. Activation of NO during abrupt cooling may be naturally induced by keeping pigeons in an unheated coop. In these birds the maximum uncoupling occurs already during the first cooling (in birds from a warm coop, during the second cooling).

3. The pattern of change of P/O during adaptation to cold conforms to the rule of biological adaptation that the range of response to a stimulus decreases as adaptation to the stimulus progresses (cf. the variability of P/O at stages I and III).

The fact cannot be ignored that during prolonged adaptation there is a certain reversal of the original

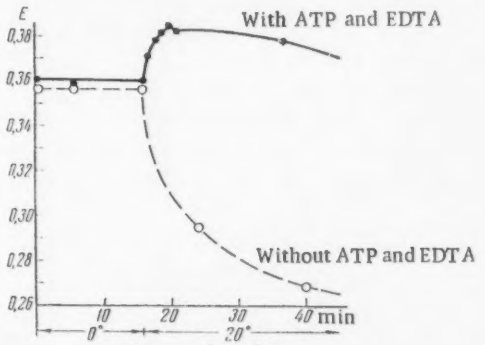

Fig. 4. Effect of ATP and EDTA on optical density of mitochondria at 0° and 20°.

changes. To be specific, in the transition to stage III the uncoupling is decreased. This was also observed by Panagos, Beyer, and Masoro in experiments on rat liver mitochondria. Mitochondria from the liver of rats kept in the cold 15 days showed a reduction of 26% in P/O, while those from rats kept in the cold a month showed a reduction of 16% [19]. Our data on muscle mitochondria resemble these data in the pattern of change of P/O during adaptation. However, the rate in which uncoupling occurs is different in the two cases. In liver, it develops after a fairly long period of acclimatization to cold (in the experiments of Panagos, et al., after two weeks; in the experiments of Smith and Fairhurst, after a month and a half).

During the first days of cold exposure, the P/O of rat liver mitochondria was the same for the cooled and the control groups. In our experiments, a short-term cooling of pigeons induced no changes in P/O of liver mitochondria [26].

In muscles a considerable reduction is often observed as early as 15-20 minutes after the beginning of cooling, and the entire cycle of thermoregulatory shifts in P/O is completed in a few days, i.e., earlier than any shift at all was noted in liver. Thus, in liver uncoupling is a feature of acclimatization, "slow adaptation" (according to Potter's terminology), while in muscle uncoupling should be related to "fast adaptation." Probably the slow changes in liver are a manifestation of a general reorganization of metabolism under the new temperature conditions and are, perhaps, the result of thyroid hyperfunction during prolonged cooling [27]. As shown in our laboratory, P/O of liver mitochondria is extremely sensitive to hyperthyreosis [28].

On the other hand, NO activation in muscles during short-term cooling may represent a special thermoregulatory mechanism for emergency heat production. As Neifakh and Zdrodovskaya showed, administration of DNP to rabbits induces a burst of heat production and an increase in body temperature; a study of P^{32} incorporation in vivo and determination of P/O in vitro revealed uncoupling of oxidation and phosphorylation [9, 15-17]. These experiments were performed at fairly high temperatures, and the excess heat production in response to DNP was injurious to the organism, a fever condition being developed. During cooling, however, evidently even the unphysiological uncoupling induced by DNP is favorable. Our experiments showed that DNP administration increases the survival of mice during cooling at -15°:

Injection	No. of expts.	Survival, min.
Without injection	25	162.8 ± 14.2
Physiological solution	29	170.3 ± 8.7
DNP	41	252.7 ± 13.0

In this investigation we have encountered physiological thermoregulatory uncoupling, which may be explained thus. The shift of oxidation to the nonphosphorylating pathway during cooling results in inactivation of the complex phosphorylating mechanism of energy accumulation, which retards oxidation and limits it to the internal zone of mitochondria. It is also important that NO does not depend on P_{inorg} levels and those of its acceptors. The excess O_2 consumption by an animal in the cold must necessarily lead to disappearance of tissue P_{inorg} after a few minutes of heightened oxidation if the level of coupling is not decreased or if there is no activation of processes degrading phosphorylated products. An acceleration of the decomposition of ATP, the product of PO, may actually occur in response to cooling. ATP is consumed during tetanic contraction of muscle in the cold.

In contrast, our uncoupling during short-term cooling is a more radical method of oxidative heat production which proves to be independent of mechanisms of ATP formation and utilization. It could be supposed that in the final stage of adaptation, the whole mechanism of increased heat production in the cold reduces to a simple combustion of extra substrate by NO enzymes, which is expressed externally as heightened consumption of nutrients and O_2.

Comparison of thermoregulatory uncoupling with other methods of uncoupling. The correlation of thermoregulatory uncoupling with changes in optical density of mitochondria and in rate of hydrogen transfer by mitochondrial DPN indicates its connection with mitochondrial structure. It might be assumed that NO activation in the cold involves a "spatial uncoupling" of complexes of the respiratory chain from phosphorylating enzymes. In this respect our observations contradict those of Smith and Fairhurst, who were unable to detect changes in mitochondrial swelling in association with P/O reduction in the liver of rats which had been exposed to the cold for a month and a half or more. This contradiction may be resolved in terms of the difference between mechanisms of thermoregulatory uncoupling in muscle during short-term cooling and slow uncoupling in liver during acclimatization to cold.

Of interest is the fact that the effect of a number of biological compounds known as NO activators is associated with change in mitochondrial structure similar to that observed on cooling. Lehninger's recently discovered natural uncoupling factor of mitochondria also induces swelling. The fall in optical density of mitochondria and in rate of oxidation through DPN accompanies uncoupling induced by calcium ions and thyroid hormones. The inhibition of oxidation is reduced by addition of ATP and/or EDTA [25, 28-30].

On the other hand, uncoupling poisons of the DNP type (phenol derivatives, coumarin, gramicidin) are unable to induce mitochondrial swelling [31]; ATP and EDTA cannot reverse their uncoupling effect. These facts are summarized in Table 4, based on experiments in our laboratory and that of Lehninger [25, 31].

The well known fact of activation of hormone production by the thyroid gland during cooling [27] could have been taken to indicate a similarity of uncoupling mechanisms during hyperthyreosis and cooling. Thermoregulatory uncoupling differs strikingly from the phenomena attendant, on thyroid hyperfunction, however. A comparison of certain characteristics of the organism's condition during various types of uncoupling revealed the following (based on our data and those of Martinus [3] and Neifakh [16, 17]):

	Cooling	Hyperthyreosis	DNP-induced fever
Uncoupling in the liver	–	+	+
Uncoupling in muscle	+	–	Not investigated
Time required for uncoupling	Ten minutes	Weeks	Hours
Gas exchange	Increased	Increased	Increased
Condition of organism during uncoupling	Normal	Pathological	Pathological

Analysis of results of experiments with ATP (Table 1-3) does not bear out the assumption of an ATPase mechanism of reduction of phosphorylation during cooling. If ATP had been degraded by ATPase during cooling, then ATP added to mitochondria from pigeons which had been subjected to cooling would have been dephosphorylated, and the P_{inorg} content of the samples with ATP should have been greater than that of the samples without it. Experimentally, the reverse was true: ATP sharply enhanced phosphate esterification. This "coupling" effect of ATP is also incompatible with an assumption of an ATPase mechanism for thermoregulatory coupling, since no cases of ATPase inhibition by catalytic amounts of ATP are known. It seems improbable, moreover, that activation of ATP dephosphorylation would be so evident with the method of P/O determination employed: Phosphorylation was measured in the presence of an excess of hexokinase and glucose to accept ATP. It is known that this system competes successfully with ATPases. Finally, direct measurement of ATPase activity showed that cooling of an animal is substantially without effect on the rate of ATP dephosphorylation by mitochondria.

Potter's hypothesis [18] of a "thermogenic shunt" at the pyridine nucleotide stage is invalid, inasmuch as uncoupling is demonstrated not only with substrates oxidized through DPN, such as pyruvic and malic acids, but also with succinic acid. Evidently all phosphorylation stages in the respiratory chain are subject to uncoupling.

These data may provide a sufficient basis, it seems to us, for the assumption that there is an in vivo uncoupling mechanism similar to that induced by dinitrophenol and thyroxin, although it differs from them both.

Probably the mitochondria operate by a vigorous mechanism which ensures a rapid dissociation of the respiratory chain from phosphorylation processes. If so, it is difficult to believe that emergency mobilization of heat is the sole function of NO.

SUMMARY

A study of oxidative phosphorylation of mitochondria of skeletal muscles as well as of the body temperature and gas exchange in pigeons subjected to abrupt transient cooling has disclosed the following three stages of rapid-adaptation to cold. 1. Decrease of the P/O coefficient, transient increase in the gas exchange and development of hypothermy of the body upon first cold exposure. 2. Further decrease in P/O, steady increase in gas exchange and stable body temperature upon second cold exposure. 3. Partial normalization of P/O and of gas exchange upon further adaptation to cold.

The degree of uncoupling of oxidation from phosphorylation at various stages is correlated with the swelling of mitochondria and inhibition of hydrogen transfer by the mitochondrial DPN. Oxidation via DPN becomes normal upon addition of DPN to the reaction mixture while P/O and optical density are increased by addition of ATP and EDTA.

TABLE 4. Comparison of Thermoregulatory Coupling with Other Types of Coupling in Vitro

Indicator	Cooling	Ca^{++}, thyroxin	DNP
Mitochondrial swelling	Activated	Activated	Inhibited
Oxidation through DPN	Inhibited	Inhibited	Unchanged
Reversal by ATP and EDTA of swelling and uncoupling	Reversed	Reversed	Not reversed

The mechanism of uncoupling upon cold exposure differs enzymatically from the dinitrophenol uncoupling and is similar to that caused by thyroxine and Ca ions. However, contrary to thyreotoxicosis, upon transient cooling uncoupling is noted in the muscle but not in the liver and progresses much more rapidly.

Rapid and powerful activation of the non-phosphorylating oxidation correlating with the survival on cold is considered as evidence that the oxidative reactions not coupled with phosphorylation are of major metabolic importance and participate in thermoregulation: nonphosphorylating oxidation in the muscle tissue is a thermoregulatory mechanism of emergency mobilization of heat.

We are deeply grateful to Professor S. E. Severin for his constant attention, valuable advice, and help in this study.

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CHANGES IN AMINO ACID COMPOSITION OF TISSUE PROTEINS OF THE DEVELOPING SILKWORM LARVA

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The necessity of a systematic survey of amino acid composition of insect proteins, especially of changes occurring during ontogenesis, has long since become apparent. It becomes particularly obvious with examination of patterns of amino acid metabolism in the silkworm.

In the period prior to formation of the cocoon, i.e., mainly during the second half of the fifth instar, there is a rapid accumulation of amino acids and proteins. This involves both uptake and incorporation of amino acids from the nutrient supply, and synthesis of amino acids in the body of the silkworm larva. Quantitative assessments of these processes have been made [1], but very little is known as to the ultimate fate of these amino acids, i.e., in what organs and in what amounts they are incorporated.

As of now it is very difficult to evaluate the rate of amino acid metabolism and protein synthesis from tissue to tissue or the relative amount of synthesis in a given tissue. It is wholly obscure whether amino acid accumulation in a tissue or organ occurs as a result of a generalized increase in its proteins, or whether only some of the proteins characteristic of the given tissue or organ are synthesized. An attempt to resolve this problem has been made in this study.

METHODS

Material to be analyzed was fixed in 75% alcohol (final concentration) at the beginning (just after melting) and the end (before cocoon formation) of the fifth instar. The following tissues and organs were removed from the silkworm larva for study: hemolymph, fat body, muscles, intestinal wall, silk gland wall, and silk gland contents. Preparation of the larvae was carried out as carefully as possible* so that data obtained might be utilized for calculations on the basis of 100 individuals. Material from 32-36 individuals was taken at the beginning of the instar and from 23-25 individuals at its close. Samples were stored as alcoholic preparations until use.

The tissue or organ was ground in a mortar with 75% alcohol to extract free amino acids, the brei then filtered on a Buchner funnel, and the solid residue washed several times with 75% alcohol. This residue was dried in a vacuum drying oven at 40-60°, weighed, again ground, and assayed for water content. Dry weight of the solid material obtained from the tissue or organ in this manner was calculated from these data. The fine powder obtained was used for further analysis.

The filtrates were made up to a volume of 50-300 ml, depending on amount of filtrate, and assayed for dry matter content. To these values were added the values for dry weight of the solid residue to give a total dry weight for the given tissue or organ.

Alcoholic extracts were assayed for total nitrogen by the Kjeldahl method, and for amino acids. In amino acid determinations, aliquots of from 0.02000 to 0.02500 g were simultaneously hydrolyzed with 400 volumes of 20% HCl (glass distilled) for 24 hours; the reaction was carried out in sealed ampullae with constant agitation of the mixtures. A slight residue of humins was filtered off on a Buchner funnel and the hydrolyzate was vacuum dried at 35-40°. The dry residue was taken up in water acidified with HCl, a small amount of humins being removed by filtration. Amino acids in the samples thus obtained were determined by paper chromatography [2].

RESULTS AND DISCUSSION

From the data obtained, we calculated amounts of individual amino acids in the various tissues and organs, absolute dry weights, and nitrogen contents. Results of the calculations are presented in Tables 1, 2, and 3.

There are some published reports of the amino acid composition of tissues of silkworm and a number of ether insects. Examination of these data reveals that opportunities for drawing comparisons with our data are extremely limited.

In insects other than silkworms, only proteins of the cuticle and of certain secretions, i.e., of tissues we

*We are indebted to Professor N. N. Sinitskoi for the gift of the larvae, and to V. K. Kondrat'evaya, N. A. Teplits, and E. P. Yakushkinaya, for assistance in removal of tissues and organs.

TABLE 1. Absolute Dry Weight (G/100 Individuals) and Nitrogen Content (%) of Tissues and Organs of the Silkworm Larva

Tissue or organ	Absolute dry weight		Nitrogen	
	1st day of 5th instar	before spinning of cocoon	1st day of 5th instar	before spinning of cocoon
Intestinal wall	4,10	10,70	14,62	13,88
Muscles	2,18	10,95	15,47	13,10
Fat body	1,92	22,30	14,16	6,35
Hemolymph	3,28	12,13	8,29	12,66
Silk gland	0,25		13,76	
Wall of silk gland		2,30		16,52
Contents of silk gland		12,22		17,62

TABLE 2. Content of Bound Amino Acids in Tissues and Organs of the Silkworm Larva at the Fifth Instar (In % of absolute dry wt.)

Amino acids	Intestinal wall		Musceles		Fat body		Hemolymph		Silk gland	Wall of silk gland	Contents of silk gland
	A*	B*	A	B	A	B	A	B	A	B	B
Glycine	3,71	3,82	4,13	3,89	3,41	1,32	2,27	3,39	5,05	8,70	16,64
Alanine	3,81	4,31	4,31	4,03	4,31	1,54	1,51	2,79	5,09	15,07	28,75
Valine	3,57	3,45	3,92	3,84	3,62	1,71	2,12	4,54	3,26	2,59	1,52
Leucine and isoleucine	7,91	7,73	8,19	8,01	7,49	3,15	3,00	7,17	7,35	5,66	2,39
Aspartic acid	4,31	5,02	5,32	5,10	4,40	1,64	3,85	5,81	4,84	4,61	4,74
Glutamic acid	8,32	8,16	9,08	8,79	7,97	2,07	3,63	7,64	7,61	5,61	3,03
Serine	3,55	3,77	3,63	3,42	3,59	1,27	2,14	2,98	3,89	5,58	7,26
Threonine	3,70	3,79	3,57	3,53	3,57	1,42	1,88	3,68	4,25	3,01	1,87
Cystine and cysteine	2,30	1,91	1,36	0,87	1,18	0,61	1,45	2,12	0,99	0,69	0,51
Arginine	4,95	4,86	5,52	5,11	4,88	1,70	2,35	4,46	4,48	4,74	4,52
Lysine	5,08	5,02	5,84	5,87	5,32	2,10	5,79	6,23	4,65	3,93	1,39
Histidine	2,50	2,26	2,27	2,04	2,16	0,82	1,03	1,98	1,92	1,98	1,87
Proline	4,68	5,00	5,06	4,62	5,46	2,70	3,72	6,09	4,77	3,62	3,24
Phenylalanine	7,75	7,81	8,32	8,03	7,41	3,60	2,98	7,40	6,76	5,26	2,56
Tyrosine	2,55	3,09	3,01	3,07	2,86	1,31	1,58	4,00	2,87	3,70	5,67

*Here and in Tables 3 and 4 A – larvae on the first day of the 5th instar; B – larvae before spinning of the cocoon.

have not examined, have been analyzed for amino acid content. In silkworm, the amino acid composition of hemolymph proteins has been studied in detail, and there is some material on amino acid composition of the silk gland [3-7]. In neither silkworm nor other insects, however, has amino acid composition of the intestinal wall, muscles, or fat body been investigated. Unfortunately the attempt of Kallhammer [8] to study amino acid composition of various organs of immature bees was not further exploited. Moreover, even a comparison of our data with those of other workers on hemolymph and silk gland of silkworm is rendered difficult by the fact that analyses were made at different stages of development and that modes or preparation of tissues were different.

If, nonetheless, these differences are ignored, it can be concluded that amounts of various amino acids in proteins of silkworm hemolymph measured by other workers are extremely close to our values; only leucine plus isoleucine, aspartic acid, glutamic acid, and cytine plus cysteine differ markedly in content. On the other hand, there is no similarity between ribonucleoproteins from the silk gland or proteins of the silk gland wall with respect to amino acid composition. In all probability this is because of the presence in the wall of silk profibroin, which has a very specific amino acid makeup.

Examination of the data in Table 3 on amino acid composition of proteins from various tissues reveals a general similarity among proteins of the intestinal wall, muscles, fat body, hemolymph, and silk gland taken as a whole, while proteins from the wall and the contents of the silk gland taken just before spinning of the cocoon have a very characteristic composition. In the first case, there are significant amounts of leucine plus isoleucine, glutamic acid, and phenylalanine (8-10%),

TABLE 3. Amino Acid Content of Proteins from Tissues and Organs of the Silkworm Larva of the Fifth Instar (In % of protein with 16% nitrogen)

Amino acids	Intestinal wall		Muscles		Fat body		Hemolymph		Silk gland	Wall of silk gland*	Contents of silk gland**
	A	B	A	B	A	B	A	B	A	B	B
Glycine	4,06	4,40	4,27	4,75	3,85	3,33	4,39	4,29	5,87	8,70	16,64
Alanine	4,17	4,96	4,46	4,92	4,87	3,88	2,92	3,53	5,91	15,07	28,75
Valine	3,90	3,98	4,06	4,69	4,09	4,32	4,10	5,74	3,80	2,59	1,52
Leucine and isoleucine	8,65	8,91	8,47	9,78	8,46	7,94	5,80	9,06	8,55	5,66	2,39
Aspartic acid	4,72	5,79	5,51	6,23	4,98	4,14	7,43	7,34	5,63	4,61	4,74
Glutamic acid	9,10	9,41	9,39	10,73	9,00	5,23	7,00	9,66	8,85	5,61	3,03
Serine	3,88	4,35	3,75	4,18	4,05	3,19	4,12	3,77	4,53	5,58	7,26
Threonine	4,05	4,37	3,70	4,31	4,04	3,58	3,62	4,66	4,94	3,01	1,87
Cystine and cysteine	2,51	2,21	1,40	1,06	1,34	1,53	2,79	2,68	1,15	0,69	0,51
Arginine	5,42	5,60	5,71	6,25	5,51	4,28	4,54	5,64	5,21	4,38	4,52
Lysine	5,56	5,78	6,04	6,95	6,01	5,29	11,17	7,87	5,41	3,93	1,39
Histidine	2,74	2,60	2,35	2,49	2,44	2,06	1,99	2,50	2,23	1,98	1,87
Proline	5,12	5,76	5,23	5,64	6,17	6,79	7,19	7,70	5,54	3,62	3,24
Phenylalanine	8,48	9,78	8,60	9,81	8,37	9,07	5,76	9,35	7,88	5,26	2,56
Tyrosine	2,80	3,56	3,11	3,75	3,23	3,30	3,05	5,06	3,33	3,70	5,67

*In protein with 16.52% nitrogen.

**In protein with 17.62% nitrogen.

TABLE 4. Amino Acid Content of Proteins in Various Tissues and Organs of the Silkworm at the Fifth Instar (In grams dry wt. for 100 individuals)

Amino acids	Intestinal wall		Muscles		Fat body		Hemolymph		Silk gland	Wall of silk gland	Contents of silk gland
	A	B	A	B	A	B	A	B	A	B	B
Glycine	0,15	0,41	0,09	0,43	0,07	0,29	0,07	0,41	0,01	0,20	2,03
Alanine	0,16	0,46	0,09	0,44	0,08	0,34	0,05	0,34	0,01	0,35	3,51
Valine	0,15	0,37	0,09	0,42	0,07	0,38	0,07	0,55	0,01	0,06	0,19
Leucine and isoleucine	0,32	0,83	0,18	0,88	0,14	0,70	0,10	0,87	0,02	0,13	0,29
Aspartic acid	0,18	0,54	0,12	0,56	0,08	0,37	0,13	0,70	0,01	0,11	0,58
Glutamic acid	0,34	0,87	0,20	0,96	0,15	0,46	0,12	0,93	0,02	0,13	0,37
Serine	0,15	0,40	0,08	0,38	0,07	0,28	0,07	0,36	0,01	0,13	0,89
Threonine	0,15	0,41	0,08	0,39	0,07	0,32	0,06	0,45	0,01	0,07	0,23
Cystine-cysteine	0,09	0,20	0,03	0,10	0,02	0,14	0,05	0,26	0,003	0,02	0,06
Arginine	0,20	0,52	0,12	0,56	0,09	0,38	0,08	0,54	0,01	0,11	0,55
Lysine	0,21	0,54	0,13	0,62	0,10	0,47	0,19	0,76	0,01	0,09	0,17
Histidine	0,10	0,24	0,05	0,22	0,04	0,18	0,03	0,24	0,005	0,05	0,23
Proline	0,19	0,53	0,11	0,51	0,10	0,60	0,12	0,74	0,01	0,08	0,40
Phenylalanine	0,32	0,84	0,18	0,88	0,14	0,80	0,10	0,90	0,02	0,12	0,31
Tyrosine	0,10	0,33	0,07	0,34	0,05	0,29	0,05	0,49	0,01	0,09	0,69

while histidine, cystine, and cysteine are present in small amounts (1-2.8%); the remaining amino acids each represent 4-5% of the total. In the second case, the amino acid composition reflects the character of silk proteins being synthesized; there are large amounts of glycine, alanine, serine, and tyrosine, while the other amino acids are present only in slight amounts.

The data presented in Tables 2 and 3 provide evidence of changes in proteins of the tissues investigated during development of the larva in the fifth instar. In view of the fact that the over-all composition of tissues and organs changes to a considerable extent during development, while the amino acid content of protein with 16% nitrogen represents an arbitrary value obtained

TABLE 5. Change in the Amino Acid Composition of Proteins from Various Organs and Tissues of Silkworm During the Period from the First Day of the Fifth Instar to the Time Just Prior to Spinning of the Cocoon (in %)

Amino acids	Intestinal wall	Muscles	Fat body	Hemolymph	Wall of silk gland*	Contents of silk glands *
Glycine	1,9	2,6	—15,0	—15,4	82,1	193,2
Alanine	7,5	4,9	—12,7	— 1,9	218,8	407,0
Valine	— 7,8	0;2	11,5	13,4	—45,4	— 72,6
Leucine and isoleucine	— 3,4	4,9	2,7	25,5	—40,8	— 79,1
Aspartic acid	11,9	0,2	— 4,9	—22,4	0,2	— 16,2
Glutamic acid	— 4,5	3,0	—37,0	11,8	—40,8	— 73,3
Serine	— 0,4	1,9	—17,9	—25,8	18,4	28,6
Threonine	1,9	4,7	— 6,2	8,2	—36,2	— 66,8
Cystine and cysteine	—17,2	—28,5	43,7	—25,0	—39,3	— 71,1
Arginine	— 4,0	0,2	—13,3	— 2,6	0,2	— 20,6
Lysine	— 4,1	2,4	— 3,5	—42,3	—18,0	— 75,4
Histidine	—10,4	— 5,6	— 7,6	15,4	— 8,9	— 33,6
Proline	4,1	— 0,4	23,2	—11,0	—27,1	— 42,2
Phenylalanine	— 1,9	4,9	17,2	29,9	—45,4	— 77,6
Tyrosine	23,1	4,3	19,1	41,4	—18,0	— 0,3

*In relation to the whole gland.

by calculation and not taking into account the nitrogenous material insoluble in 75% alcohol, it is appropriate to evaluate change in proteins on the basis of amounts of the individual amino acid components.

The appropriate calculations, based on the data of Tables 1 and 2, are presented in Table 4. If we take the ratio of amino acid content before spinning of the cocoon to that on the first day of the fifth instar, the value obtained will indicate the average increase in the given amino acid.

The minimum increase, about three-fold, was found in the intestinal wall. In the muscles, fat body, hemolymph, and silk gland, bound amino acids increased 5-10-fold. The greatest increase, 70-fold, was observed in the contents of the silk gland.

This increase is accompanied by changes in the proportionate amounts of individual amino acids. If the increase in each amino acid is expressed as a percentage of the over-all increase in amino acids of a given tissue or organ, a set of values is obtained which characterizes the change in proteins of that tissue or organ. Results of such calculations are presented in Table 5.

In the muscles and intestinal wall, the proportionate amounts of amino acids show changes which are on the average within the limits of error (5-7%); in the fat body and hemolymph these changes are 2-3 times in excess of these limits, and in the wall of the silk gland and especially in its inner contents they become extremely high (Table 5).

It is evident that in the course of development of a fifth instar larva the protein composition of muscles and intestinal wall does not undergo substantial alteration; the only change is one of quantity of protein, which increases with organ growth. In contrast, in the fat body, hemolymph, and silk gland there is not only a considerable accumulation of proteinaceous material, but also an extensive reorganization of protein. Amino acid composition alters with larval development, with new proteins characteristic of the given period of growth being synthesized and laid down.

With the data of Table 4, it is possible to trace the accumulation of bound amino acids during development of the fifth instar larva. Knowing the amount of amino acid laid down during this period [1], the relative amount accumulated in a given tissue can be calculated. Such a calculation reveals that the main portion is incorporated in the silk gland and the hemolymph, while in the other tissues there is a lower level of incorporation, no marked differences being noted from tissue to tissue.

These conclusions as to the lability of proteins in various tissues of the silkworm are in good agreement with those based on other methods. For example, a quantitative determination of changes in protein fractions of the hemolymph of a fifth instar larva, and of certain amino acids in these fractions indicated a high rate of protein turnover in this tissue [9]. Investigation of the fate of nitrogen in various tissues of silkworm, including hemolymph, also revealed that hemolymph proteins play a not inconsiderable role in supplying the silk gland with amino acids; in males, proteins of the fat body also serve as a source of supply, while the integument, muscles, and gonads are inactive in this respect [10]. Incorporation of S^{35}-methionine into proteins of the silk gland wall, hemolymph, and fat body is considerably greater than into muscle and intestinal wall proteins [11]. Although differences among tissues in incorporation of C^{14}-glycine were not so marked, the

same order of activity was found: silk gland contents, silk gland wall, hemolymph, fat body, intestinal wall. In the experiments of Sisakyan et al. [12-17] with silkworm pupae, the important role of the hemolymph in protein and amino acid metabolism during metamorphosis was firmly established; this is supported by a study of the metabolism of other materials, especially carbohydrates, in silkworm hemolymph [18].

In an assessment of protein metabolism of various silkworm tissues, notice should be taken of results of many workers who studied enzymatic activity of these tissues.

Simura et al. [19] showed that the silk gland contains no proteinase, while it has active transaminases [20] and a phosphoamidase [19]. There are a number of reports of proteases in the hemolymph of the fifth instar silkworm larva, including those with a pH optimum in the acid range and those with an optimum in the alkaline range [21-24]; a vigorous nuclease has also been reported [24]. Of special interest, however, are data on the comparative activity of enzymes involved in protein and amino acid metabolism in the various tissues [25-32]. These enzymes are particularly active in the silk gland and the intestinal wall, fairly active in the fat body, and weakly active in the hemolymph, even though here all the proteolytic enzymes characteristic of the other tissues are also present. Possibly this is a reflection of the fact that the hemolymph is to a certain extent a way station in the transfer of proteins and amino acids from the digestive tract and the fat body to the silk gland. Nevertheless, it should be emphasized that the role of the hemolymph cannot be reduced merely to one of transport. It possesses a unique nitrogen metabolism, which plays a not inconsiderable part in silkworm development, especially in the critical periods, larval molting, spinning of the cocoon, metamorphosis. In this connection, an enzyme characteristic of hemolymph is the "pH 5 enzyme", which mediates incorporation of amino acids into protein [33].

Although our experimental data do not permit us to say that changes in the amino acid composition of hemolymph proteins are the result of degradation and synthesis in the hemolymph itself rather than movement of proteins from the intestinal wall or the fat body, we are inclined to this point of view on the basis of published reports. We do not, however, completely reject the alternative view. Probably both processes occur. There is no doubt that the high activity of enzymes in the intestinal wall is related primarily to digestion and that other aspects of protein metabolism are considerably less important.

Thus, the pattern of enzyme activities associated with protein and amino acid metabolism in various tissues of the silkworm shows a general correlation with patterns of protein reorganization in these tissues which were elucidated in this laboratory.

SUMMARY

Within certain limits, the quantitative amino acid composition of proteins of a number of silkworm tissues (intestinal wall, muscles, fat body, hemolymph) shows little variation. Some other tissues, however, have a distinctive composition. For example, proteins of the silk gland are characterized by high levels of alanine, glycine, serine, and tyrosine, and by minimum levels of cystine plus cysteine, lysine, and valine. Prior to cocoon formation, hemolymph proteins contain much tyrosine, while at the beginning of the fifth instar they are rich in lysine and relatively poor in phenylalanine and leucine plus isoleucine. In the fat body, glutamic acid is low prior to cocoon formation.

In the course of larval development, during the fifth instar, not only the amounts of bound amino acids, but also their relative proportions, change in the silk gland, hemolymph, and fat body. There is little change in relative amounts of amino acids in the intestinal wall and the muscles.

Protein metabolism is most rapid in the silk gland and hemolymph, somewhat less rapid in the fat body, and sluggish in the muscles and intestinal wall.

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COMPARISON WITH RESPECT TO NUCLEOTIDE COMPOSITION OF DESOXYRIBONUCLEIC ACIDS ISOLATED FROM INSECTS AND MAMMALS BY VARIOUS METHODS

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In elucidation of the chemical specificity of nucleic acids as related to phylogenesis, it is important to know the identity and sequence of the component nucleotides. However, there are no suitable methods for determining sequence at the present time, and we are limited to an investigation of composition.

A large number of studies have been devoted to the composition of DNA and RNA from various sources, but information relating to the whole range of living types is far from complete in this respect. Nucleic acids of insects have been almost completely neglected, there being only scattered references to the nucleotide composition of insect DNA. This can be attributed to the difficulty of isolating DNA from insect tissues in a form suitable for analysis.

In 1951, Wyatt [1] reported on the nucleotide composition of grasshopper DNA. According to his data, the coefficient A + T/G + C, which expresses the molar ratio of component nitrogen bases (A – adenine, T – thymine, G – guanine, C – cytosine) or of nucleotides and is characteristic of DNA of a given species [2], proved to be 1.42. This "specificity coefficient" differed from those of other DNA's (1.30* for ox DNA 1.27 – sheep, 1.33 – rat, 1.23 – herring, 1.52 – sea cucumber, 1.15 – wheat) [1]. In another study Wyatt [3] reported on the nucleotide composition of DNA of silkworm and nun-moth, pointing out that the DNA's of insects differ from those of their infecting viruses. In this study no quantitative data were included.

Sisakyan and Gumilevskaya [4] determined the composition of DNA of eggs from silkworm pupae. Its specificity coefficient was 1.26. Material analyzed was unpurified DNA obtained from dried tissues by the method of Spirin and Belozerskii [5].

Preliminary results of a determination of the nucleotide composition of DNA from silkworm, oak-moth, and the house fly have been reported [5]. The high-polymer DNA's were isolated from whole silkworm pupae and fly larvae by a variant of Gulland's method, using sodium citrate at all stages of isolation [6]. Specificity coefficients of 1.43 and 1.49 were obtained with different preparations of silkworm.

Published information on the inhomogeneity of DNA [7-9] necessitate measures to ensure that all the DNA in a given tissue be isolated, as far as this is possible. We therefore extracted tissues several times [6, 10] and, moreover, thoroughly washed dry tissue with acid prior to extraction and then suspended it in alkali; finally, extracted total DNA was not purified before analysis [4, 5]. A change in extraction method consisting of omission of the preliminary washing out of acid-soluble phosphorus of dried bacteria [11] represented an improvement in isolation conditions, since acid medium affects DNA composition. Results of preliminary experiments showed, however, that DNA preparations obtained by the method of Spirin and Belozerskii [5, 11] from silkworm pupae were strongly contaminated with substances which interfere with subsequent chromatographic separation of nitrogen bases. As Bendich indicates [7], heating of powders in a solution of NaCl instead of base results in precipitation of proteins, while nucleic acids go into solution. With this procedure, we were able to obtain purer preparations from silkworm.

This communication is concerned with the method of isolation of DNA from insects and mammals and analysis of its nucleotide composition.

METHODS

Material. Pupae and starved larve of the third to fifth instars of silkworm and oak-moth, and also house fly larvae, were frozen in liquid air or in a freezer. Mice and rats were decapitated and their organs quickly removed and frozen. Frogs and some of the mice were frozen whole (without skin and alimentary tract). Organs of the ox (calves, cows) were frozen immediately after sacrifice of the animals, being transferred from the chopping block to ice. The organs of many animals were pooled for isolation of DNA. Frozen tissues were minced with shears.

*Calculations of coefficients from published data (here and elsewhere) are ours.

METHODS OF ISOLATION OF DNA

Method 1. Isolation according to one of the variants of Gulland's method [6]. Tissues and organs which had been in a frozen condition for 30 minutes to 1 hour were homogenized in 0.14 M NaCl with citrate cooled to 0°; after several washings with the same solution, the solid material was centrifuged off. Nucleoproteins were extracted three times with 2 M NaCl containing citrate for 48 hours each time. After reprecipitation with ethanol, the nucleoprotein was dissolved in 2 M NaCl with citrate and freed of protein by treatment with chloroform and isoamyl alcohol. DNA was then precipitated with ethanol.

Method 2. Powders containing unpurified DNA from dried tissue were prepared by the method of Spirin and Belozerskii [5, 11] without preliminary washing out of acid-soluble phosphorus.

Method 3. Powders containing unpurified DNA were obtained according to Bendich [7]; extraction of nucleic acids was carried out in 2 M NaCl adjusted to pH 8.

Method 4 (combined). Frozen minced tissue was homogenized twice in a blendor with 96% ethanol cooled to 0°, then twice in a cold mixture of ethanol and ether (1:1 by volume), then twice in ether, the solid residue being removed by centrifugation. The air-dried tissue was ground to a powder and an aliquot extracted and repeatedly washed with 10-20 volumes (for tissues with low DNA content, 5-10 volumes) of 2 M NaCl, pH 8, for 1 hour at 100°. Nucleic acids were precipitated in the cold with three volumes of ethanol, and the precipitate was hydrolyzed with 0.5 N NaOH for 18 hours at 37°. DNA was precipitated with chloric acid adjusted to pH 3-3.5, all solutions and containers being kept very cold. The precipitate was thoroughly washed twice with cold 0.2 N chloric acid, ethanol, a mixture of ethanol and ether, and ether. The air-dried powders (DNA preparations) were then analyzed.

Method 5. High-polymer DNA was obtained according to Hammarsten [12], with nucleoproteins being extracted with water and without the use of nuclease inhibitors.

Method 6. This is the old method of Neiman, described by Steudel [13], in which tissue hydrolysis with hot alkali leads to partial destruction of DNA and also to inhibition of nuclease activity.

ANALYTICAL AND PHYSICOCHEMICAL METHODS

Determinations of DNA, RNA, phosphorus, protein, and molecular weight of DNA were made as previously described [6]. Nucleotide composition of DNA was investigated by chromatography [14]. Chromatograph paper was thoroughly washed with 2% HCl [15] or with 2 M acetic acid, then with water, and then dried. Each hydrolyzate was applied to 5-7 parallel paper strips. Preparations were analyzed after several days or even a week, since analysis of parallel aliquots on the same day usually yields identical results. Experimental and control strips, after examination in UV (light filter UFS-1) were cut into equal parts containing spots of nitrogen bases. Extinction of the eluates was measured on an SF-4 spectrophotometer against the solvent, 0.1 N HCl). Extinction of the control eluates was subtracted from extinction of the corresponding experimental eluates to minimize the error from unequal distribution of contaminants remaining after washing of the strips. Calculations of amounts of nitrogen bases were made according to Spirin and Belozerskii [5].

RESULTS

High-polymer DNA preparations from insects and mammals had the form of fine white threads clumped into balls. The admixture of RNA comprised less than 1%, of protein, 0.6-0.7%; water content was ~ 20% (preparations dried in air). Molecular weight of DNA from silkworm pupae was 1.0-1.5 million, from mice, 4-4.5 million.

DNA preparations and powders containing unpurified DNA, which were obtained by methods 2, 3, 4, and 6, were white, gray or yellowish in color. The viscosity of their solutions was low. Only preparations from the thymus, obtained by method 6, gave viscous solutions, but their viscosity was 60 times lower than that of solutions of high-polymer DNA.

Data on the yield of DNA-containing powders obtained by methods 2-4, and on their content of DNA salts as compared with that of preparations obtained by method 6, are presented in Table 1. The yield of DNA-containing preparations made from dry tissues of silkworm by method 2 is higher than that of preparations made by methods 3 and 4, but the DNA concentration is lower, i.e., they are more contaminated. In this case, method 4, gives the best result, since preparations made by this method have the highest DNA concentration. (DNA concentration plays a large role in subsequent chromatographic analysis.)

A similar DNA preparation could not be obtained from dried tissues of oak-moth pupae by method 4, probably because of the very low DNA concentration (less than 20 mg%) [16]. There was very little DNA in preparations from dry liver tissue of mice and frogs, the nitrogen bases being hardly distinguishable on chromatograms. Powders from dry tissues of mouse and rat spleen and kidney and calf thymus had a lower DNA concentration than did high-polymer preparations. DNA obtained from insects and frogs by method 6 was more impure.

According to method 4, after alkaline hydrolysis ribonucleotides were washed with weak acid and then with alcohol. In attempting to precipitate these nucleotides from alcohol with ether, we obtained powders with the following "DNA salt" concentrations (determined according to Webb): 2.6% for silkworm, and 1.6% for thymus. The reaction employed indicated the presence

TABLE 1. Yield of Preparations Made by Various Methods and Their Content of the Sodium Salt of DNA

Source	Methods of isolation of DNA	Preparation No.	Yield from 4.0 g dry tissue mg	Na-DNA, %
Silkworm:				
pupae	2	104[a]	19	5,6
"	3	104[b]	12	6,2
"	4	104	6	39,3
"	6	1 & 3	—	0,3--2,4
Oak-moth:				
pupae	3	99[b]	8	Little
"	4	99	27	1,1
"	6	6	—	1,5
Mice:				
liver	2	118[a]	10	Little
"	3	118[b]	4	
"	4	118	5	
spleen	4	119	36,6	35,0
kidneys	4	120	44,0	34,6
Rats:				
kidneys	4	112	—	33,3
spleen	4	115	—	36,0
Calves:				
thymus	2	28[a]	620	55,3
"	3	28[b]	1280	36,9
"	4	28	1100	46,0
"	6	5		72,3
Frogs:				
whole	4	27	65,6	3,0
"	6	25	—	13,1

TABLE 2. The Effect of Two Methods of Washing Chromatographic Paper on Determinations of Molar Ratios of DNA Nitrogen Bases

DNA source	Preparation No.	Acid used in washing papers							
		HCl				acetic			
		$\frac{G}{C}$	$\frac{A}{T}$	$\frac{A+G}{C+T}$	$\frac{A+T}{G+C}$	$\frac{G}{C}$	$\frac{A}{T}$	$\frac{A+G}{C+T}$	$\frac{A+T}{G+C}$
Silkworm	53	1,00	0,99	0,99	1,44	0,99	0,97	0,97	1,61
Oak-moth	124	0,95	0,94	0,95	1,61	0,99	0,98	0,98	1,76
Mice	47	0,99	0,97	0,98	1,35	0,99	0,95	0,97	1,44
Rats	61	1,01	0,92	0,96	1,32	0,97	1,00	0,99	1,47
Ox	64	1,06	1,02	1,04	1,24	1,07	0,96	0,99	1,35

of desoxypentoses. Upon chromatographing hydrolyzates of the powders, we found weak spots for guanine, adenine, cytosine, and thymine, but no uracil. This compels the conclusion that, despite all precautions, treatment with acid entails a small acid hydrolysis of DNA.

In the course of the study it became clear that the method of washing chromatographic paper is very important in obtaining reproducible results of nucleotide analysis. Table 2 shows that while it makes little difference whether the paper is washed with HCl or acetic acid as far as the ratios G/C, A/T, or A + G/C + + T are concerned, the specificity coefficient does vary with washing agent. The values were consistently somewhat higher when acetic acid was employed. Probably they are closer to the true values, since acetic acid is more effective in washing out "chromatographic contaminants". In further presentations of results of chromatography, therefore, the method of washing is indicated.

Results of analysis of DNA preparations obtained by various methods are summarized in Table 3. In making up the table, we confirmed previous reports [17-19] and [26], touching on DNA of carp, ox, mice, and rats, that DNA's from various organs of a single animal are identical in nucleotide composition.

Table 3 shows that DNA's of silkworm, mice, rats, and ox obtained in high-polymer form by method 1 and in unpurified from by method 4, have practically identical nucleotide compositions, confirming to Chargaff's rule. The small acid hydrolysis occurring during treatment by method 4 is not reflected in nucleotide composition, possibly because chromatographic analysis is not very accurate. The specificity coefficients for DNA

TABLE 3. Nucleotide Composition of DNA Isolated by Various Methods

DNA source	Preparation No.	Method	Nitrogen bases, mole %				G/C	A+G/C+T	A+T/G+C washing agent	
			G	A	C	T			HCl	CH_3COOH
Silkworm:										
whole	117	1	19,0	30,3	19,3	31,4	0,98	0,97	—	1,61
"	104[b]	4	18,7	31,1	19,4	30,8	0,96	0,99	—	1,62
"	3	6	7,3	21,9	26,6	44,2	—	—		—
Oak-moth:										
whole	123	1	18,0	31,5	18,2	32,3	0,99	0,98	—	1,76
"	6	6	6,2	25,3	27,8	40,7	—	—		
House fly:										
whole	8	1	20,3	30,1	18,9	30,7	1,07	1,01	1,56	—
"	7	6	12,8	22,4	39,2	25,6	—	—		
Ox:										
liver	64	1	22,0	28,2	20,5	29,3	1,07	1,00	—	1,35
thymus	28	4	21,4	28,5	21,3	28,8	1,00	0,99	—	1,34
"	36	5	20,9	29,5	20,5	29,1	1,02	1,01	—	1,42
"	15[a]	6	22,0	27,2	22,4	28,4	0,98	0,97	1,25	
"	28	4	23,1	27,6	21,3	28,0	1,08	1,03	1,25	
liver	64	1	23,0	28,0	21,6	27,4	1,06	1,04	1,24	
Rats:										
liver	61	1	19,9	29,7	20,6	29,8	0,97	0,98	—	1,47
spleen	115	4	20,9	29,6	20,2	29,3	1,03	1,02	—	1,43
Mice:										
whole	47	1	21,1	28,3	21,4	29,2	0,99	0,98	1,35	
"	26	4	22,0	27,9	20,8	29,3	1,06	1,00	1,34	
"	x**	1*	20,5	29,5	20,5	29,5	1,00	1,00	—	1,44
"	32	1[a]	18,8	29,4	21,7	30,1	0,87	0,93	—	1,47
Frogs:										
whole	25	6	17,1	25,7	24,8	32,4	—	—	—	—
"	27	4	Spots poorly visible				—	—	—	—

*Method 1[a] differs from method 1 in temperature employed during isolation.

**Average values from analyses of nine different preparations.

of insects are consistently higher than those for DNA of the mammals studied, as shown in Tables 2 and 3.

If conditions of isolation by method 1 are not strictly adhered to, the composition of DNA deviates from Chargaff's rule. The G/C value for preparation No. 32 from mice (Table 3), which was obtained under temperature conditions other than those prescribed by the method, was reduced to 0.87; this value was obtained in repeated determinations. Isolation of DNA from calf thymus by method 5 yielded a preparation with ratios of nitrogen bases characteristic of native DNA, but the specificity coefficient in this case was consistently higher than values obtained by analysis of DNA's isolated by methods 1 and 4. Method 6 proved to be wholly unsuitable for obtaining DNA from insects or frogs; ratios of nitrogen bases of such preparations were extremely distorted; and therefore have not been presented. This method yields preparations from calf thymus, which had a high DNA content, with the same nucleotide composition as that of preparations obtained by methods 1 and 4.

On chromatograms of hydrolyzates of DNA obtained from insects by methods 2 and 3 and from oak-moth, frogs, and mouse liver by method 4, the spots for nitrogen bases were hardly visible, and cytosine and thymine were often completely undetectable, even though the size of aliquot taken for hydrolysis was increased. The small quantities of hydrolysis products make it difficult to detect bases on the paper and reduce the accuracy of further analysis. In the analysis of preparation 110, for example, the total micromoles of base fluctuated from 0.120 to 0.140 on six chromatograms. The coefficients G/C, A/T, and A + G/C + T averaged ~ 1 (1.00-1.02), but the specificity coefficients were much below those usually found for the species in question. The total amount of bases optimal for analysis of nucleotide composition of DNA is 0.8-1.5 micromoles.

DISCUSSION

This study has shown that in the isolation of DNA by methods which ensure maximum extraction from tissues and minimum destruction, molar ratios of the component bases conform to Chargaff's rule, i.e., G/C, A/T, and A + G/C + T are ~ 1. Repeated determinations have shown that the average value of these ratios

TABLE 4. Molar Ratios of Nitrogen Bases of DNA as Obtained by Various Workers

Source of DNA	No. of preparations	Molar ratios* $\frac{G}{C}$	$\frac{A}{T}$	$\frac{A+G}{C+T}$	$\frac{A+T}{G+C}$	Special features of isolation or analysis	Author
Silkworm	—	1,03	1,05	1,04	1,25	Isolated acc. to [5]	[4]
	2	0,95	0,96	0,96	1,46	High-polymer DNA paper washed with HCl	[6]
	3	0,98	0,99	0,98	1,61	Isolation by methods 1 and 4. Paper washed with CH_3COOH	This study
Mouse	5	0,76	0,80	0,78	1,55		[20]
	4	0,94	0,95	0,95	1,45	Method 1. Paper washed with CH_3COOH	[10]
	9	1,00	1,00	1,00	1,44		This study
Rat	7	0,72—1,00	0,78—1,30	0,81—1,22	1,15—1,35	Various methods. Hydrolysis in HCOOH	[21]
	1	1,08	1,08	1,12	1,21		[22]
	2	1,00	1,00	1,00	1,33	Hydrolysis in HCOOH	[1]
	3	1,11	1,02	1,05	1,46		[19]
	2	1,00	1,01	1,01	1,45		This study
Ox	15	Close to		1,00	1,26—1,31	Isolation without inhibition of nuclease. Hydrolysis in HCOOH	[1]
	4	Close to		1,00	1,28—1,31	Isolation without inhibition of nuclease. Hydrolysis in HCl. Separation on starch column	[23]
	21	1,00	1,02	1,00	1,36		[24]
	6	0,99	1,01	1,00	1,37	Paper washed with CH_3COOH	[19]
	3	1,05	1,01	1,02	1,35		This study

*Calculated in several cases from published data.

is exactly one (Table 3, analysis of nine preparations of DNA from mice).

Data obtained in this laboratory and elsewhere on molar ratios of nitrogen bases from DNA's of various origins are presented in Table 4. Our data on nucleotide composition of ox DNA are similar to those of Chargaff [24] and Zbarskii [19], which were obtained by similar methods of isolation and analysis. The specificity coefficient for ox DNA isolated without protection from nuclease action, and hydrolyzed with formic or hydrochloric acid, proved to be different [1, 23]. Probably, in this case failure to inhibit nuclease played a part, so that some of the adenine and cytosine was destroyed during treatment with formic acid. That such destruction could occur has been indicated [25]. Results of our analysis of rat DNA are in agreement with those of Abarskii [19], except that we did not find an increased G/C ratio. Wyatt [1] obtained a lower specificity coefficient than ours. This is probably associated with his use of formic acid in hydrolysis. The values of G/C, A/T, and A + G/C + T obtained by some workers [21, 22] are very far from one; this can be attributed to peculiarities in the methods of isolation and analysis employed in these studies. The same thing is true of the deviant values for mouse DNA found by Schneider [20]. It is not surprising that our value for the specificity coefficient of mouse DNA [10, this study] differ from that of Schneider. The discrepancy in specificity coefficients of silkworm DNA obtained in this laboratory and elsewhere† is probably associated with the low DNA concentration of the preparations obtained by the method of Spirin and Belozerskii [5] from insect tissues.

These and other data bear witness to the fact that the methods of isolation and analysis are of great significance in assessing the nucleotide composition of DNA's from various sources. Conformity to Chargaff's rule is one of the main criteria of correctness of method, though not the only one.

SUMMARY

Certain methods of DNA isolation have been studied along with procedures which increase accuracy

of chromatographic analysis of nucleotide composition. The method of DNA analysis without isolation from tissues (silkworm pupae and certain organs of mammals) proposed by the authors gives the same results as analysis of purified high-polymer DNA preparations: Molar ratios of nitrogen bases obey Chargaff's rule for native DNA, the "specificity coefficients" A + T/G + C being similar in both cases. These "specificity coefficients" of insect DNA (silkworms, domestic fly) are higher than the respective coefficients of mammalian DNA (ox, rat, mouse). Inadequate methods of isolation and certain features of this procedure which damage DNA change nitrogen bases ratios of isolation DNA so that they do not obey Chargaff's rule.

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† After preparation of this paper, there appeared a study by Zbarskii et al., (Zhur. Obshchei Biol. 20, 428, 1959) in which the average specificity coefficient for silkworm DNA was given as 1.43, which is close to our value.

‡ Original Russian pagination. See C. B. translation.

ON THE PROPERTIES OF AN ENZYME SYSTEM OF RAT KIDNEY WHICH CATALYZES AMINO ACID SYNTHESIS FROM PYRUVIC ACID AND AMMONIUM SALTS

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The enzyme system which catalyzes amino acid synthesis from pyruvic acid and ammonia in kidney is found not only in the mitochondria, as in liver, but also in other cell organelles in almost the same concentration [1]. The distribution of this system in kidney cells therefore differs considerably from that in liver cells. It was of interest to find out whether it differs from the liver system in other ways as well. It seemed of primary importance to study its electrophoretic properties, since Berezovskaya [2] was able to isolate the enzyme in purified form from liver mitochondria by starch electrophoresis. A second objective of our study was to separate the enzyme system in question from a transaminase also present in kidney, as Berezovskaya [3] was able to do for the corresponding enzymes in liver mitochondria.

METHODS

Rat kidney homogenates were fractionated in the same manner as previously described [1]. The fraction obtained after removal of mitochondria by centrifugation of 20% homogenates at 12000 rpm for 10 min served as the enzyme source. This fraction comprised supernatant liquid plus microsomes. Since its activity was comparatively low, we concentrated it in a number of cases by lyophilization and suspension of the residue in a small volume of phosphate buffer. It was established in separate experiments that this procedure does not noticeably reduce enzyme activity. The original or concentrated fraction was subjected to electrophoresis on starch at a potential gradient of 6 v per cm and a current of 20 ma for 16-18 hours. At the conclusion of electrophoresis the starch block was cut into separate strips and each strip was extracted with potassium phosphate buffer. In the eluates, obtained as previously described [1], rate of amino nitrogen synthesis from pyruvic acid (final concentration, 80 μM per sample) and ammonium carbonate (60 μM per sample) was determined. Total protein was determined by Lowry's method in separate samples and rates of synthesis were calculated on the basis of 1 mg protein nitrogen.

In those experiments in which parallel determinations of transamination rate were made, transaminations α-ketoglutaric acid and alanine and between α-ketoglutaric and aspartic acids were measured. In both cases, the increase in glutamic acid was measured, using Giri's method [4], after separation by paper chromatography (solvent system, butanol – acetic acid – water).

RESULTS

A study of the electrophotretic distribution of protein nitrogen of the supernatant liquid containing microsomes revealed that the maximum amounts exceeding 500 μg per sample,* were concentrated in fractions 7-9. As Table 1 shows, synthesis of amino acids from keto acids is concentrated mainly in fractions 5-6, i.e., in the fractions adjacent to the site of application (fraction 7) on the side toward the anode. In the majority of experiments (7 of 11), 70-100% of all the activity was concentrated in this fraction. In other experiments, a more or less perceptible activity was sometimes observed in fractions 1-2 and 3-4, i.e., in fractions closest to the anode. More frequently, activity could be detected in fractions 8-9, 10-11, i.e., in fractions next to the cathode. If these data are compared with those of Berezovskaya on the electrophoretic separation of extracts disrupted liver mitochondria, it is easy to see that according to electrophoretic mobility the kidney fraction containing activity differs markedly from that of liver. Only fractions 1-2 and 3-4 from the liver, counting from the anode, contain activity; in those

*In fractions 1-6 the content of protein nitrogen at the edge is low – 20 μg or less; in certain cases a large quantity of protein nitrogen is observed; in fractions 10-11, lying close to the cathode, where it constituted 90 μg of protein nitrogen per sample.

TABLE 1. Synthesis from Keto Acids and Ammonia of Amino Nitrogen in Fractions of a Microsome-Containing Supernatant Liquid Which Were Obtained by Starch Electrophoresis (In μM amino acid per mg nitrogen)

Anode	1—2	3—4	5—6	7*	8—9	10—11	12—13	Cathode
Pyruvic acid								
	30	13	2,8	—	0,6	29	0,3	
	0	0	17,1	—	0	0	0	
	20	25	0	—	4,4	2,2	0,14	
	0	0	47	4,33	2,7	11,2	—	
	0	0	96	—	1,1	19	—	
	0	0	0	—	0,37	1,1	—	
	0	0	55	—	0,63	0,54	0	
	0	0	38	—	0	1,87	—	
	0	0	20	—	0,6	0,28	—	
	0	0	16	—	0,66	—	—	
α-Ketoglutaric acid								
	—	0	74	—	0,9	10	—	
	—	0	97	—	1,5	8,8	—	
	0	0	38	—	2	0,52	—	
	0	0	51,5	—	0,48	0,14	0	
	0	0	—	—	1,1	1,4	—	
	—	0	9	—	2,0	0,81	0	
	—	0	31	—	0,77	1,3	—	
	0	0	0	—	—	1,9	—	

*Site of application.

TABLE 2. Rate of Transmination Between Aspartic and α-Ketoglutaric Acids (I) and Between Alanine and α-Ketoglutaric Acid (II) in Fractions Obtained by Starch Electrophoresis of Microsome-Containing Supernatant Liquid Expressed as Increase in Glutamic Acid in μM per mg N

Expt. No.	Transaminase	Anode	Fractions					Cathode
		1—2	3—4	5—6	7*	8—9	10—11	12—13
1	I	0	0	0	—	2,6	55,4	101
	II	0	0	0	—	6,6	3,8	7,4
2	I	0	0	0	—	35	56	250
	II	0	0	0	—	28	6	77
3	I	0	0	0	—	24	23	67
	II	0	0	0	—	5,2	4	1,6

*Site of application.

fractions migrating toward the cathode, no activity was ever observed.

These data on synthesis of amino acids from a keot acid were obtained with pyruvic acid as a substrate. It was of considerable interest to localize by starch electrophoresis another amino acid-synthesizing system, namely glutamic dehydrogenase, which catalyzes synthesis of glutamic acid from α-ketoglutaric acid (Table 1).

Distribution of this enzyme among the fractions obtained by starch electrophoresis was the same for that of the system mediating synthesis from pyruvic acid. Distribution of glutamic dehydrogenase among kidney fractions is, therefore, different from that associated with liver mitochondria fractions; in the latter case, activity was found mainly in the zone of greatest transamination activity, i.e., in fraction 7-8, located in the immediate vicinity of the site of application.

In this connection, it was possible partially to separate the enzyme mediating direct amination of pyruvic acid from glutamic dehydrogenase in mitochondrial extracts; in our kidney fractions, however, this

separation could not be effected. This indicates that amination of pyruvic acid and ketoglutaric acid in the hyaloplasm and microsomes of kidney is accomplished by enzymes which possess similar electrophoretic mobilities.

The situation differs with respect to separation of the enzyme for pyruvic acid amination from those mediating transamination between aspartic and ketoglutaric acids and between alanine and ketoglutaric acid. Data touching on this are presented in Table 2.

As these data show, both transaminases are found only in the fractions moving toward the cathode (fractions 8-9, 10-11, 12-13). In not a single experiment was transaminase activity found in those fractions containing the enzyme for amino acid synthesis from pyruvic acid, i.e., in fraction 5-6.

It has therefore been possible to separate clearly the enzyme mediating amino acid synthesis from pyruvic acid from the chief transamineses. It should be pointed out that in all transaminase-containing fractions the activity of the glutamic-oxalacetic transaminase is considerably higher than that of the glutamic-pyruvic transaminase. This is especially clear in fractions 10-11 and 12-13, where for the most part only glutamic-oxalacetic transaminase is present.

CONCLUSIONS

The activity of an enzyme system catalyzing amination of pyruvic acid in a microsome-containing supernatant obtained by differential centrifugation of a rat kidney homogenate proves to be associated with a protein fraction moving toward the anode during starch electrophoresis. Only a small part of the activity, not more than 30% moves toward the cathode

Glutamic dehydrogenase is concentrated in these same fractions, as determined by rate of synthesis of amino acid from α-keotglutaric acid. In this connection, the microsome-containing supernatant obtained from kidney homogenates differs from mitochondrial extracts of rat liver, in which the enzyme catalyzing synthesis from pyruvic acid can be partially separated from glutamic dehydrogenase by electrophoresis.

In the kidney preparation, the enzyme catalyzing synthesis from pyruvic acid can be separated from glutamic-pyruvic and glutamic-oxalacetic transaminases.

In such a preparation, glutamic-oxalacetic transaminase activity is considerably higher than that of glutamic-pyruvic transaminase.

I wish to thank Professor S. Ya. Kaplanskii for his unflagging direction and assistance in this study.

SUMMARY

Amino acid synthesis from keto acids and ammonia has been followed up in the microsome-containing supernatant fraction obtained by differential centrifugation of a rat kidney. Partition of these fractions by starch electrophoresis showed that amino acid synthesis from pyruvic acid is most rapid in fractions migrating towards the anode.

In the same zone glutamic dehydrogenase, which catalyses synthesis of glutamic acid from α-ketoglutaric acid, showed its highest activity. The enzymes of transamination between aspartic and α-ketoglutaric acids and between alanine and α-ketoglutaric have been found only in fractions migrating toward the cathode. Thus, by means of electrophoresis, it is possible to separate completely in the above structural elements of the kidney the enzyme catalyzing alanine synthesis from pyruvic acid, from the glutamic-pyruvic and glutamic-oxalacetic transaminases.

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ON THE CONTENT OF ADENOSINE PHOSPHORIC ACIDS IN LIVER AND MUSCLES OF DINITROPHENOL-POISONED RATS

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In the course of a study of adenosine phosphoric acid metabolism in rats with alloxan-induced diabetes [1], we made a comparative study of rats poisoned with dinitrophenol (DNP) and found changes of opposite nature in the relative specific activity (RSA) of the terminal phosphate of ATP in liver and muscles. In liver, it was reduced almost twofold, while in muscles it was somewhat increased. It was of interest to determine whether these differences in nucleotide metabolism between liver and muscle of DNP-poisoned animals involved only the rate of metabolism of terminal phosphate of ATP, or whether they also involved levels of adenosine phosphoric acids in these organs.

The effect of DNP, one of the strongest uncoupling agents, has been studied in vitro in detail with preparations from various tissues, and also in vivo, using whole microorganisms. In vivo studies of the disruption of animal metabolism associated with DNP administration are considerably fewer in number. We have investigated changes in nucleotides, mainly adenosine phosphoric acids, in liver and muscle of DNP-poisoned rats.

METHODS

Experiments were carried out on white rats weighing from 180 to 250 g. A group of animals (10-15 rats) was administered 0.25% DNP in 0.1% $NaHCO_3$ by intraperitoneal injection on the basis of 25 mg DNP per kg weight. Control experiments were performed on groups of normal rats. Several minutes after DNP administration the animals exhibited shortness of breath and loss of mobility and they refused food. After 1 to 2 hours, they were sacrificed while under barbamyl narcosis, the barbamyl being administered 5 minutes prior to killing on the basis of 50 mg per 200 g weight. Immediately afterwards the chest and abdominal cavities were opened, the heart slit, the blood rapidly drained off, and the liver or muscles removed. These were frozen with dry ice or liquid air, ground in a mortar, weighed, and extracted with an equal volume of cold 10% TCA. The mixture was centrifuged, the supernatant filtered off, the solid residue extracted a second time with half a volume of 4% TCA and centrifuged; the supernatant was filtered off and pooled with the main filtrate.

Nucleotides were adsorbed from the TCA filtrates directly onto OU GOST 4453-48 activated charcoal, "Carbolene", using 1 mg charcoal per unit extract measured at 260 mμ [2]. The charcoal was separated by centrifugation, thoroughly washed with dilute TCA and then with water, and eluted twice with 40% ethanol containing ammonia, elution being carried out with shaking [3]; at each elution, the eluant volume was equal to the volume of the original TCA filtrate. Extent of adsorption and elution was determined by measurement of extinction at 260 mμ. Yield after two successive elutions was usually equivalent to ~ 80% E_{260}. Since it was increased only very slightly by a third elution, this was omitted. Ethanol was added to the eluate to a final concentration of 48% (some of the experiments with liver) or 72% (remaining experiments). Nucleotides were precipitated as the barium salts with barium acetate, the salts being removed by centrifugation and dried over P_2O_5 in a desiccator.

Nucleotides were separated with an anion exchange resin AB-17 or a Dowex-1 in the chloride form. In most of the experiments, 50 to 93 μM of nucleotide (700 to 1300 extinction units), or sometimes less, depending on yield, was applied to an 8 cm × 1.1 cm^2 column. Nucleotides were first converted to the soluble sodium salts by treatment of the barium salts with a solution of Na_2SO_4. For separation of adenosine phosphoric acids on an AB-17 column, we used a previously described method [4], which is easily modified to obtain a large number of fractions. Solutions of the same concentration were used for eluting Dowex-1 and AB-17 columns. Elution was followed by measuring extinction at 260 mμ. Each peak was taken off as a separate fraction; those samples with maximum extinction were subjected to determination of UV absorption spectra at pH 2. For isolation of fractions with an extinction of not less than 10 units, contents of tubes were pooled, the extreme tubes discarded, and neutralization to pH 7 carried out as rapidly as possible, fractions were stored in a refrigerator until resumption of column separation procedures, which took 3-5 days. Nucleotides were freed of salts and concentrated by a second adsorption on activated

TABLE 1. Adenosine Phosphoric Acid Content (in % total E_{260}) of a Nucleotide Fraction, Precipitated in the Form of Barium Salts in the Presence of 48% Alcohol, from Liver of Normal and DNP-Poisoned Rats. In experiments with poisoned animals, they were killed 1 hour after administration of DNP

Expt. No.	Exptl. cond.	ATP	ADP	AMP
1	Control	26	29	8,0
2	"	18	46	9,0
3	"	23,8	41	5,6
4	DNP poisoning	2,7	13,5	49,4
5	"	5,0	35	19,4
6	"	5,8	29	14

charcoal. For this, solutions were acidified once again to pH 2 and absorbed under the same conditions as those used in the isolation of the total nucleotide fraction (1 mg charcoal per extinction unit); columns were eluted with the same eluting solution three times, each time on the basis of 1 ml per ten extinction units for fractions with not less than 100 units; fractions with lower nucleotide content were eluted with increased amounts of eluting fluid, so that for those fractions with the lowest extinctions it was not less than 3 ml for each elution. Average recovery was 80%, although in a number of experiments it was considerably lower as a result of losses during preparation, chiefly during centrifugation with charcoal. Alcoholic solutions of the fractions were vacuum distilled at 30-35° to dryness. For identification of the fractions and determination whether the individual peaks represented single substances or mixtures, fractions were dissolved in distilled water in volumes from 0.2 ml to 3 ml, depending on concentration, and chromatographed on paper in five solvent systems: 1) phosphate – isoamyl alcohol [5]; 2) isobutyric acid – NH_3 – versene [6]; 3) phosphate – $(NH_4)_2SO_4$ – n-propanol [7]; 4) ethanol – ammonium acetate, pH 7 [8], with versene and oxyquinoline added; 5) n-propanol – NH_3 – H_2O [9], with versene and oxyquinoline. In the last two cases, separation was continued for two days, after which a new portion of solvent, but without versene and oxyquinoline, was added to the tray and separation was continued for two days longer, according to the method of Venkstern and Baev [10].

Procedures employed in paper chromatography have been described earlier [4]. Chromatographically pure preparations of ATP, ADP, AMP, DPN, IMP, UMP, and UDP served as markers. Other nucleotides were identified by the position of peaks during elution from a column, by UV absorption spectra, by fluorescence (guanine and pyridine nucleotides), by nitroprusside and enzyme reactions (CoA), and by Rf values obtained in various solvent systems. In several experiments additional tests for more complete identification were carried our, as for example treatment with bromine water.

TABLE 2. Content of Adenosine Phosphoric Acids (in % of Total E_{260}) of Nucleotide Fraction, Precipitated in the Form of Barium Salts in the Presence of 72% Alcohol, from Liver and Muscle of Normal and DNP-Poisoned Rats. In experiments with poisoned animals, they were killed 2 hours after administration of DNP

Expt. No.	Exptl. cond.	ATP	ADP	AMP
	Liver			
7	Control	13.5	26.3	29.4
8	"	15.0	30.7	23.7
9	DNP poisoning	Traces	14.4	51.6
10	" "	2.7	20	50.7
11	" "	1.8	19	38.5
	Muscles			
12	Control	67.6	14.0	2.6
13	"	62.3	17.5	4.2
14	DNP poisoning	52.9	25.7	5.5
15	" "	53.3	20.6	5.4

RESULTS

Results of determination of adenosine phosphoric acid content of a nucleotide fraction from liver of normal and DNP-poisoned rats are summarized in Table 1. In this series of experiments the nucleotide fraction was obtained as the barium salt in the presence of 48% alcohol. As the table shows, total content of adenosine phosphoric acids comprises from 63 to 73% of all nucleotides in a normal liver – ATP, 18-26%, ADP, 29-46%, and AMP, 5-9%.

In rats poisoned with DNP, ATP levels were sharply reduced (to 2.7-5.8%), ADP fell perceptibly only in some experiments (expt. 4), and AMP increased, but not always significantly (expt. 6). Total content of adenosine phosphoric acids in the nucleotide from liver decreased somewhat (to 49-65%). With the method employed in this series of experiments, that of precipitating nucleotides in the presence of 48% alcohol, AMP and other nucleotide monophosphates were not recovered completely. Since in DNP-poisoning the greatest increases would be expected in the AMP fraction of liver, in succeeding experiments with both liver and muscle, nucleotides were precipitated (in the form of barium salts) in the presence of three volumes of 72% alcohol. Results obtained for liver and muscle are presented in Table 2. In order to get more pronounced changes in the nucleotide fractions after DNP administration, time between administration and sacrifice of the animals was increased to 2 hours.

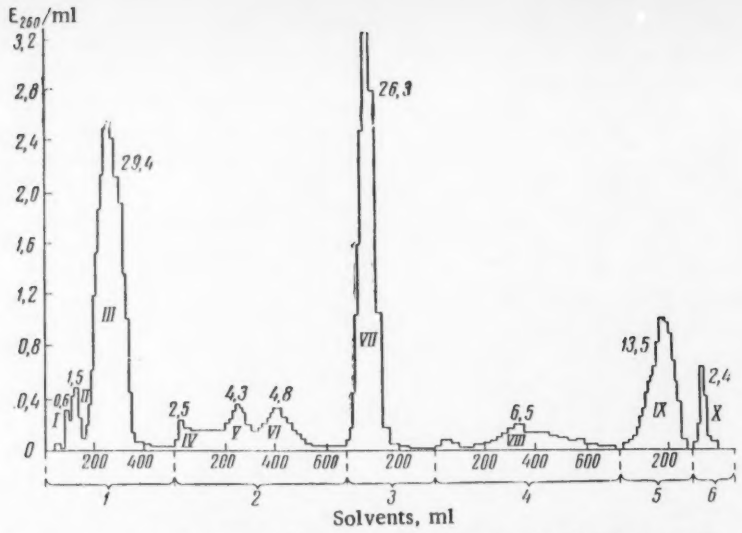

Fig. 1. Separation of individual nucleotides (nucleotide fraction precipitated in the presence of 72% alcohol) from liver of normal rats on a Dowex-1 × × 4 (Cl^-) column. Expt. 7. Eluting solutions: 1) 0.003 N HCl; 2) 0.01 N HCl; 3) 0.02 N NaCl in 0.01 N HCl; 4) 0.05 N NaCl in 0.01 N HCl; 5) 0.1 N NaCl in 0.01 N HCl; 6) 0.5 N NaCl in 0.01 N HCl. Peaks: I) CMP; II) DPN; III) AMP; IV) UMP + unidentified adenine derivatives; V) GMP; VI) unidentified compound + IMP; VII) ADP; VIII) GDP + S-S-CoA; IX) ATP; X) GTP. Figures beside peaks represent % E_{260}. Recovery – 92.7%.

The data presented in Table 2 show that when nucleotides from liver are precipitated in the presence of 72% alcohol their components are considerably altered in amount, there being an increase in the AMP fraction. In normal rats, ATP corresponds to 15% the total E_{260}, ADP to 26-31%, and AMP to 24-29%; altogether the adenosine phosphoric acids comprise 69% of the total nucleotide. In DNP-poisoned rats, ATP falls sharply (from traces to 2.7%), ADP falls an average of 38%, and AMP rises markedly, 77% on the average, comprising about half the total nucleotide. The total content of adenosine phosphoric acids is not perceptibly changed.

In the nucleotide fraction from muscle of normal rats (Table 2), the ATP content is 62 to 68% of the total, ADP – 14-17%, and AMP only 2-4%; in all, adenosine phosphoric acids make up 84% of all nucleotides. In DNP-poisoned rats, there is a small decrease in ATP, 17% on the average, some increase in ADP, 30%, and a slight increase in AMP, to 5.5%. The IMP (inosinic acid) fraction, which might have been expected to increase in muscle with DNP-poisoning, also rose slightly, as will be shown in the elution curves.

In DNP-poisoned rats, therefore, there are marked differences in liver and muscle with respect to changes in the nucleotide fraction. While the changes induced in liver were characteristic of those associated with DNP action, i.e., a marked decrease in ATP and a concurrent increase in AMP, those in muscle were slight.

In order to present a more complete picture of changes in the nucleotide fraction induced by DNP-poisoning, elution curves are shown in Figs. 1-4.

In these experiments, CMP was identified by the position of its elution peak and by its UV absorption spectrum before and after bromination (Fig. 5). DPN was identified by its blue fluorescence in UV after spraying of the chromatogram with KCN [11], by the sky-blue fluorescence after exposure of the chromatogram to vapors of an HN_3 – acetone mixture [12], and by the identity of its Rf with that of a DPN marker. Guanine nucleotides were identified by position of their elution peaks, by violet fluorescence on the chromatogram after treatment with HCl vapor [10], and by Rf. CoA was identified by the nitroprusside reaction for S-S groups in the presence of KCN [13], and also by a quantitative method [14].

In nucleotides from liver, CoA came off with GDP in the fraction eluted by 0.05 N NaCl in 0.01 N HCl, which just preceded the ATP fraction. Its recovery in this fraction was half the original amount put on the column in some experiments, but in other experiments was considerably lower. Taking into account the fact that enzymatic determination of CoA was preceded by separation of nucleotides on a column, treatment of fractions, and chromatographing on paper, all of which required 2 to 3 weeks, and also that some of these operations were carried out at room temperature, which cer-

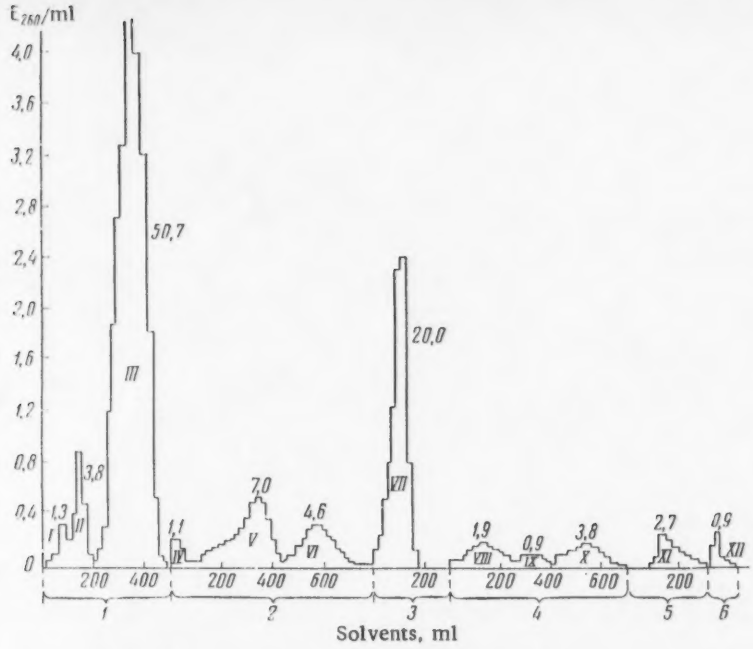

Fig. 2. Separation of individual nucleotides (nucleotides fraction precipitated in the presence of 72% alcohol) from liver of DNP-poisoned rats on a Dowex-1 × × 4 (Cl^-) column. Expt. 10. Eluting solutions – as in Fig. 1. Peaks: I-III) as in Fig. 1; IV) unidentified adenine derivative with an admixture of GMP; V) unidentified compound, not identical with IMP; VII) ADP; VIII) unidentified compound; IX) GDP + UDP; X-XII) same as VIII-X, Fig. 1. Figures beside peaks as in Fig. 1. Recovery – 90%.

tainly would not favor CoA stability, the amount of recovery in this fraction could be considered satisfactory, and this procedure could be used in isolation of CoA from a nucleotide mixture.

CoA could be detected enzymatically in the nucleotide fraction from muscle, but at a level one order of magnitude lower than in the fraction from liver. In one of the experiments it was established that CoA of muscle is eluted in the same fraction as with liver, i.e., in the one preceding the ATP fraction; in other experiments, however, the peak in question was not clearly manifested.

A comparison of Figs. 1 and 2 shows that while there are large shifts in amount of adenosine phosphoric acids in the liver of DNP-poisoned rats, other nucleotides exhibit less marked changes in amount. This lends support to the assumption of a primary role of adenosine phosphoric acids in oxidative phosphorylation reactions. Other changes were observed, however, For example, GTP content decreased, while there was corresponding increase in GMP; both CMP and DPN increased. Not a single nucleotide fraction from muscle showed significant change in amount with DNP treatment (Figs. 3 and 4); IMP (2.4 to 4.4%), CMP, DPN, and GMP increased to a slight extent, while GTP decreased.

These data concerning the basic differences, in the effect of DNP-poisoning on nucleotide of liver as compared with muscle confirm our results obtained in a determination of RSA of the terminal phosphate of ATP from liver and muscle of DNP-poisoned rats [1] and attest to the fact that nucleotide metabolism of liver is disrupted by DNP much more extensively than that of muscle. This cannot be explained in terms of a DNP-induced inhibition of oxidative phosphorylation in liver in contradistinction to muscle, since it has been shown by Dianzani and Scuro [16] that muscle mitochondria are the most sensitive to the uncoupling action of DNP.

We attribute these differences between liver and muscle [15] to a stimulation of glycolysis in muscle associated with suppression of the Pasteur mechanism by DNP, with the result that there is a considerable compensatory resynthesis of ATP. Anaerobic processes are much less vigorous in liver, and such compensation does not occur.

SUMMARY

The changes in content of adenosine phosphoric acids and of other liver and muscle nucleotides have

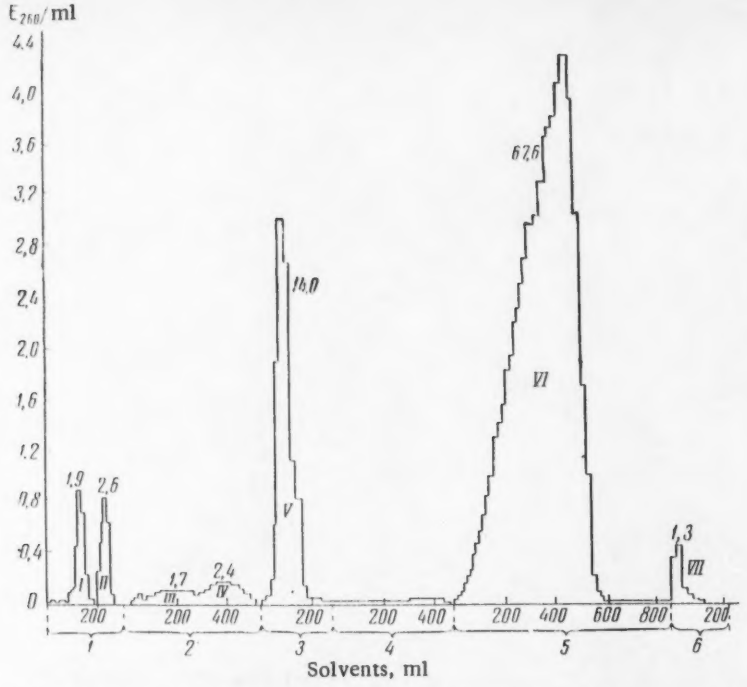

Fig. 3. Separation of individual nucleotides (nucleotide fraction precipitated in the presence of 72% alcohol) from muscle of normal rats on a Dowex-1 × × 4 (Cl^-) column. Expt. 12. Eluting solutions – as in Fig. 1. Peaks: I) DPN; II) AMP; III) GMP; IV) IMP; V) ADP; VI) ATP; VII) GTP. Figures beside peaks as in Fig. 1. Recovery – 92.5%.

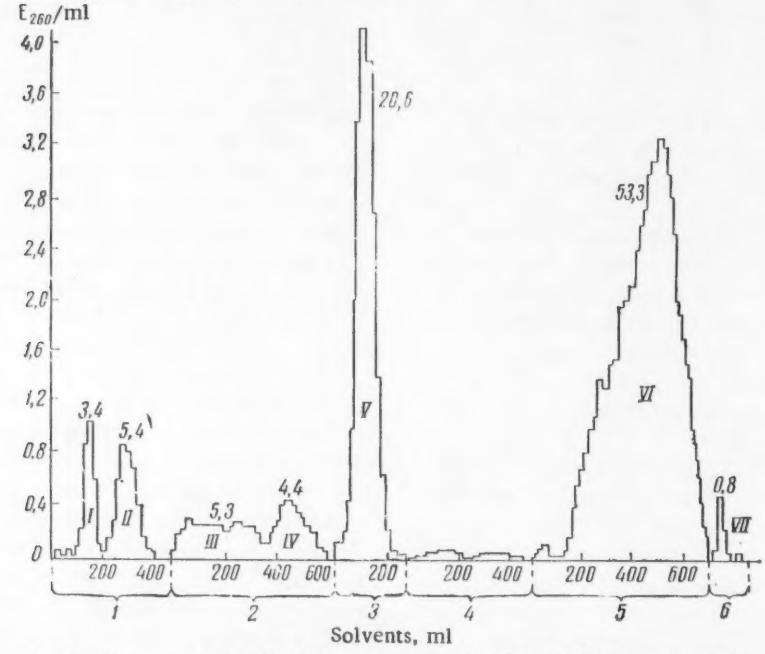

Fig. 4. Separation of individual nucleotides (nucleotide fraction precipitated in the presence of 72% alcohol) from muscle of DNP-poisoned rats on a Dowex-1 × 4 (Cl^-) column. Expt. no. 15. Eluting solutions – as in Fig. 1. Peaks: I-VII – as in Fig. 3, except for III – unidentified adenine derivative + GMP. Figures beside peaks as in Fig. 1. Recovery – 94.7%.

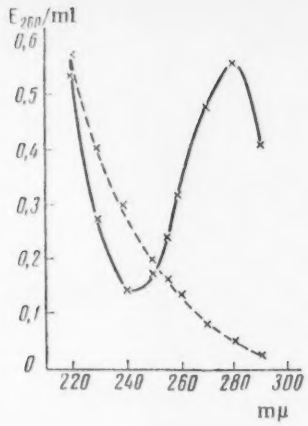

Fig. 5. UV absorption spectra of a CMP fraction before and after bromination. Dotted line – after bromination.

been traced in DNP-poisoned rats.

In the nucleotide fraction of rat liver, DNP poisoning results in a drastic decrease in ATP, a certain decrease in ADP and an appreciable increase in AMP, while the change in other nucleotides is less apparent.

However, in the nucleotide fractions of muscles of DNP poisoned rats, no appreciable changes were noted, except a slight decrease in ATP, some increase in ADP and an insignificant increase in AMP and IMP.

The causes of the differences in behavior of liver and muscle nucleotides of DNP-poisoned rats are discussed.

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GLUCOSE TRANSPORT INTO TISSUE CULTURE CELLS

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It is known that tissue culture cells have a very high clycolytic activity. Consequently, glucose must pass into these cells at a similarly high rate. It is also known that the surface layer of a number of normal cells consists of a barrier which appreciably hampers the diffusion of sugars from the surrounding medium into the cytoplasm. In tissue cultures, the properties of this surface layer, obviously, must be changed. To obtain a high rate of glucose assimilation it is necessary either to remove the barriers which interefere with diffusion or to create or develop mechanisms which will facilitate the transport of sugars into the cells. The existence of special mechanisms, capable of transporting carbohydrates through the surface of the membrane, have been shown in a number of cases, for example, in erythrocytes [1-4], tumor cells [5-7], yeasts [8], and others. We showed in our previous communication [9], that the passage of sugars into tissue culture cells had a very high temperature coefficient. This circumstance is more in keeping with the presence of sugar carriers in the membrane than with the condition of free diffusion of hexoses from the surrounding medium into the cells. However, the appreciable slowing of the transfer rate of sugars into the cells when the temperature is lowered can be explained without the transport concept. For example, it can be assumed that on lowering the temperature, the physical composition of the cell surface layer will change so sharply, that the diffusion of sugars through this layer will be significantly hampered. Thus, additional data are required in order to demonstrate the participation of carriers in the mechanism of sugar transport in tissue culture cells.

The experimental data presented in the present communication are sufficient, it seems to us, to allow us to confirm that the passage of glucose into tissue culture cells occurs by means of a special mechanism. This conclusion is mainly based on the probable competitive inhibition of glycolysis in whole cells by means of an inhibitor (galactose), which does not affect the glycolytic enzyme system in an homogenate. At any rate, we will show, that at low concentrations of glucose the tranport of this sugar into the cells limits the rate of glycolysis as a whole.

METHODS

The experiments were carried out on cells of monkey (Macacus rhesus) kidney cortex cultures (KI).* Methods of cultivating the cells, separating them from the glass surface by means of EDTA, their washing and calculations, have been described in previous communications [10, 11]. The only difference is that Krebs-Ringer-bicarbonate buffer, pH 7.4 was used for washing the cells instead of Krebs-Ringer-phosphate.

Experiments on intact cells. The rate of anaerobic glycolysis in intact cells of the tissue culutre, incubated in 3.5 ml Krebs-Ringer-bicarbonate buffer at 37° was measured manometrically by CO_2 formation in Warburg flasks. Lactate formation determined as a control by the method of Barker and Summerson [12] gave good correlation between the manometric and chemical data. At low substrate concentrations the rate of CO_2 formation decreased with time. The initial rate of CO_2 formation in these cases was taken as the rate of glycolysis. The rate of aerobic glycolysis was measured by lactate formation. A mixture of 95% N_2 and 5% CO_2 was used in an anaerobic experiment as an incubating medium; in aerobic experiments, 95% air and 5% CO_2

Experiments on homogenates. The cells were crushed in a closely-fitting ground glass Potter-Elvehjem homogenizer in the presence of a small quantity of 0.15 M KCl. The homogenate was diluted with 0.15 M KCl; the uncrushed cells, cellular fragments and nuclei were removed by centrifugation for 10 min at 1500 rpm (approx. 450 g) at 0°. A mixture of the following composition was used as an incubating medium: ATP, 10^{-3} M; DPN, 10^{-3} M; nicotinamide, 10^{-3} M; $KHCO_3$, $2.5 \cdot 10^{-2}$ M; $MgCl_2$, $3 \cdot 10^{-3}$ M; KH_2PO_4, $2.5 \cdot 10^{-2}$ M; glucose, $1.25 \cdot 10^{-2}$ to $1.25 \cdot 10^{-4}$ M; galactose, 0 to 0.125 M, pH

*The following abbreviations are used in this paper: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; G-6-P, glucose-6-phosphate; EDTA, ethylenediamine tetraacetate; TCA, trichloroacetic acid; HK, hexokinase; K_M, Michaelis constant; K_I, dissociation constant of the enzyme-inhibitor complex; KI, monkey kidney cortex tissue cultures.

7.3-7.4; total volume, 2 ml. The incubation temperature was 37°. The incubation mixture and homogenate underwent a preliminary incubation at this temperature for 10 min. The reaction was begun with the addition of homogenate to the incubation medium and was ended after 20 min by the addition of an equal volume of 5% TCA. Control determinations during different time intervals showed that the rate of glycolysis in homogenates did not vary significantly with time.

Determination of HK activity. As is known [13, 14], the HK of tissue culture cells is concentrated in the homogenate fraction not precipitated by rapid centrifugation. In order to prepare this fraction the cellular homogenate was suspended in 0.15 M KCl and subjected to centrifugation in the cold for 15 min at approx. 20,000 g. TPN reduction (increase in optical density at 340 mμ) served as a measured of HK activity in a system of the following composition [6]: ATP, $1.7 \cdot 10^{-3}$ M; TPN, $4 \cdot 10^{-4}$ M; KF, $1.7 \cdot 10^{-3}$ M; $MgCl_2$, $3.3 \cdot 10^{-3}$ M; tris buffer pH 7.95, $3.3 \cdot 10^{-2}$ M; glucose, $2.5 \cdot 10^{-2}$ to $1.25 \cdot 10^{-4}$ M; galactose, 0 to 0.25 M; supernatant fraction, 0.1 ml; total volume, 3.0 ml. The determinations were carried out in a SF-4 spectrophotometer in quartz curvettes with a light path length of 1 cm. The addition of exogenous G-6-P dehydrogenase in these experiments was not necessary, since the supernatant fraction of KI cell homogenate was found to contain a fully sufficient quantity of G-6-P dehydrogenase. Special determinations showed that the G-6-P dehydrogenase activity in the extract (determined by TPN reduction in the same system, but in the presence of $5 \cdot 10^{-2}$ M G-6-P as substrate instead of glucose) usually exceeded HK activity 6-7 times. The curve representing the change in optical density at 340 mμ with time indicated that in the first 1-3 min, the reduction of TPN occurred at a constantly increasing rate. This is related, apparently, to the accumulation of increasing quantities of G-6-P in the incubation medium (at low concentrations of G-6-P the rate of dehydrogenation of this substrate is very sensitive to changes in its concentration). After this the rate of TPN reduction became constant, and remained much lower than the maximum possible. Consequently, the rate of G-6-P formation in the course of the hexokinase reaction is equivalent to the rate of its elimination by dehydrogenation. Thus the rate of TPN reduction in this linear portion of the curve was taken as the HK activity.

Calculation of K_M and K_I. Application of the method of least squares [15] and the Michaelis equation as expressed by Lineweaver and Burk [16], permitted the calculation of the value of K_M in the following form:

$$K_M = \frac{\Sigma\left(\frac{1}{[C]}\right) \cdot \Sigma\left(\frac{1}{V}\right) - n \cdot \Sigma\left(\frac{1}{[C]} \cdot \frac{1}{V}\right)}{\Sigma\left(\frac{1}{[C]}\right) \cdot \Sigma\left(\frac{1}{[C]} \cdot \frac{1}{V}\right) - \Sigma\left(\frac{1}{[C]^2}\right) \cdot \Sigma\left(\frac{1}{V}\right)},$$

where [C] = substrate concentration, V = velocity corresponding to this concentration, n = number of determinations. In order to find K_I in the case of competitive inhibition, the same equation is used, in which V is the velocity at concentrations of substrate in the medium [C] and the concentration of inhibitor [I]. On

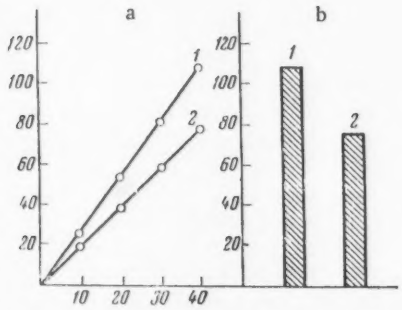

Fig. 1. Inhibition of anaerobic glycolysis by galactose in cells of monkey kidney tissue. Content of inhibitor in the tests, $8.2 \cdot 10^6$. Concentration of glucose, $1.43 \cdot 10^{-3}$ M. a) Manometric determination. Abscissa, time in minutes; ordinate, CO_2 formation in μliters. b) Chemical determination of lactate, formed after 40 min. incubation. Ordinate, formation of lactate, calculated in terms of μliters of CO_2. 1) Without galactose; 2) in the presence of 0.1 M galactose.

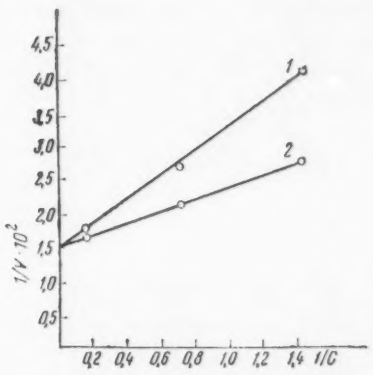

Fig. 2. Competitive inhibition of anaerobic glycolysis in cells of monkey kidney cultures by galactose. Inhibitor content in samples, $1.8 \cdot 10^6$. C, concentration of glucose in millimoles/liter. V, glycolysis in μliters of CO_2 after 60 min. 1) In the presence of 0.1 M galactose; 2) in the absence of galactose.

Aerobic Glycolysis in Homogenates of KI Cells and the Effect of Galactose on it. For Experimental conditions, see methods section

Expt. No.	Conc. of glucose, 10^{-3} M	Conc. of galactose, 10^{-3} M	Formation of lactate, μ liters	Expt. No.	Conc. of glucose, 10^{-3} M	Conc. of galactose, 10^{-3} M	Formation of lactate, μ liters
1	12,5	—	24	2	1,25	—	23,5
	1,25	—	20		0,25	—	20
	0,25	—	14		0,25	125	20
	0,25	125	16		0,125	—	11
	0,125	—	10		0,125	125	14
	0,125	125	10				

the left side of the equation, in place of K_M we obtain the quantity $K_M (1 + I/K_I)$. From this, K_I may be easily calculated, since K_M and [I] are known.

Purification of galactose. The galactose which we had at our disposal contained an appreciable glucose impurity. The galactose was purified by incubation with bakers' yeast, filtration, adsorption of the impurities on activated charcoal, evaporation and recrystallization from 80% alcohol.

RESULTS

The effect of galactose on glycolysis in intact cells. Numerous determinations have shown that anaerobic glycolysis in intact KI cells is very appreciably suppressed in the presence of galactose, which remains practically unfermented by these cells. The results of one of these experiments, in which the lactate formed was determined both manometrically and chemically, are presented in Fig. 1. The inhibitory effect of galactose was increased with decrease in glucose concentration. Kinetic analysis showed that inhibition of glycolysis by galactose was competitive (Fig. 2). Calculation of K_M for glucose gives a value of the order of $9 \cdot 10^{-4}$ M. K_I for galactose has a value of the order of 0.1 M. Galactose also inhibits anaerobic glycolysis in rabbit kidney tissue cultures (Fig. 3).

The data presented below are evidence of the fact that aerobic formation of lactic acid in KI cells in the presence of galactose was also inhibited (concentration of glucose, $1.43 \cdot 10^{-3}$ M).

Number of expt.	Quantity of inhibitor	Conc. of galactose, 10^{-3} M	Formation of lactate, μ liters	Inhibition, %
1	$2.9 \cdot 10^6$	—	35	—
		100	27	23
2	$13.4 \cdot 10^6$	—	278	—
		100	206	26

The effect of galactose on glycolysis in homogenates. Study of the effect of glucose and galactose concentrations on the aerobic glycolysis in homogenates of KI cells has shown (table) that galactose in this case did not inhibit lactic acid formation, although the ratio of galactose concentration and glucose concentration in homogenate experiments were primarily of a higher order than in experiments with intact cells. The rate of glycolysis in homogenates conformed to the Michaelis-Menten equation. Calculation of K_M for glucose gave a value of the order of $1.6 \cdot 10^{-4}$ M.

Effect of galactose on HK activity. Results of one of the experiments, in which the relationship of the hexokinase reaction rate in the supernatant fraction of the KI cell homogenate to the concentration of glucose and to the presence of galactose were determined, are presented in Fig. 4. The data verify the fact that galactose has practically no effect on HK activity. Calculation of KM for glucose gives a value of the order of $6 \cdot 10^{-5}$ M.

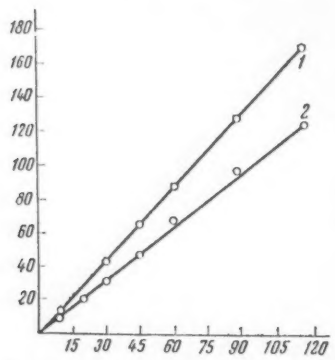

Fig. 3. Inhibition of anaerobic glycolysis in cells of rabbit kidney cultures. Inhibitor content in the sample, $2 \cdot 10^6$. Designations the same as in Fig. 1a.

DISCUSSION OF RESULTS

Method of ingress of sugars into tissue culture cells. Competitive inhibition by galactose of glucose assimilation into intact KI cells is apparently evidence of the fact that these two sugars are capable of interacting with the same active center of some biologically active compound which takes part in the assimilation of glucose. The absence of any effect of galactose on glycolysis in homogenates shows either that glycolysis in this case can take place without the participation of this compound, or that this compound so changes its structure on homogenization that its ability to react with galactose is decreased but its ability to react with glucose is completely retained. It seems to us that the first supposition is the most simple and probable. In this case it can be considered that galactose inhibits some link in the as-

similation of glucose by the cells, which preceeds the hexokinase reaction. Such a link apparently would transport sugar across the cellular membrane – a mechanism, the existence of which in different cells has been admitted by many authors. The presence in tissue culture cells of a special mechanism for the transport of sugar through the surface membrane is in good agreement with the fact that we discovered earlier that the passage of sugar into these cells has a very high temperature coefficient [9].

The question of the nature of the carrier remains quite obscure. Taking into account that the participation of HK in sugar transport across the cell membrane has been discussed repeatedly, we set up experiments to verify this hypothesis. However, the participation of HK (in every case, its active center, taking part in the phosphorylation of sugar) in the process of glucose transport can be excluded, since galactose has no effect on the activity of this enzyme. It could be supposed that the affinity of hexokinase for different sugars depends to a significant degree on the composition of the incubating medium, in particular on the presence or absence of the second partner in the hexokinase reaction – ATP.

We set up special experiments in which the KI cells were incubated in medium designed for incubating homogenates. But in this case, galactose inhibited glycolysis, which again was evidence for the difference in affinity for different sugars between HK and a hypothetical carrier. It may be noted that such a difference was also found in tumor cells [6, 17].

A rate-limiting link in the glycolysis chain, in KI cells. The fact that glycolysis in intact cells is inhibited by galactose inhibition of glucose transport is already evidence that the sugar transport rate can limit the rate of sugar assimilation. However, in order to assume that glucose transport has a regulatory function, it is important to consider the difference between K for glucose in intact cells and homogenate. Taking into account the fact we discovered earlier, that the concentration of glucose inside the KI cells is less than 10% of the glucose concentration in the incubating medium [9], we can give the following simple explanation for the differences in K_M for intact cells and homogenate. The cellular membrane consists of a barrier; the concentration of glucose on one side of this barrier (inside the cells) is considerably lower than the glucose concentration on the other side (in the incubating medium). However, a definite relationship between these two concentrations occurs; an increase in the sugar content of the incubating medium causes some increase in sugar content inside the cells (apparently this increase is not proportional). In such a case, in order to create a glucose concentration inside the cells which could saturated the intracellular glycolytic system, it is necessary to have in the incubating medium, an appreciably large concentration of this sugar. Such an explanation for the differences in K_M between the cells and the homogenate indicates that at least at low concentrations of glucose the rate of glycolysis in intact KI cells is limited by the rate of sugar transport across the cell membrane.

Our conclusions, it is understood, only apply to one culture and only to a certain region of glucose concentrations. Under different conditions and in different cultures, it is possible that different links in the process of sugar assimilation will determine the rate of glycolysis [14].

Similarity and difference of KI cells from other experimental material. Investigations in our laboratory have shown that tissue culture cells closely resemble

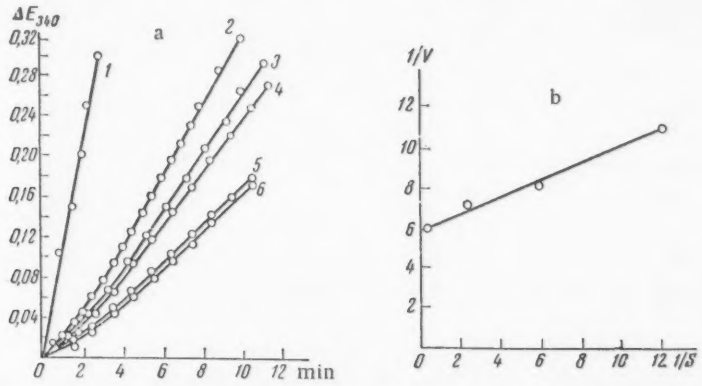

Fig. 4. Effect of glucose concentration and the presence of galactose on the hexokinase reaction rate. a) TPN reduction in the presence of: 1) $5 \cdot 10^{-2}$ M G-6-P; 2) $8.33 \cdot 10^{-3}$ M glucose; 3) $4.17 \cdot 10^{-4}$ glucose; 4) $1.67 \cdot 10^{-4}$ glucose; 5) $8.33 \cdot 10^{-5}$ glucose; 6) $8.33 \cdot 10^{-5}$ M glucose and 0.167 M galactose. b) Relationship between the hexokinase reaction rate and substrate concentration, expressed according to Lineweaver and Burk.

cells of malignant tumors in their peculiarities of carbohydrate-oxidative [10, 11] and energetic [18] metabolism, in their mitochondria content [13] and in some other properties. This permits us to assign tissue explants to a considerable group of tissues and cells, having a "cancerous" type of metabolism [19]. One of the most important peculiarities of cells in this group is high glycolytic activity, which assumes an ease of penetration of sugars across the cell membrane. Thus, there is considerable interest in studying the mechanism of penetration of different members of this group, and determining how these mechanisms differ from the passage of substances into the cells with "normal" metabolism.

Comparison of the data presented in the present communication with results of other authors in regard to the permeability of tumor cells, indicates the presence of a certain similarity in the mechanism of permeability of tumor cells and tissue explants. However, quantitative differences either in rate of sugar transport across the membrane, or in the rate of sugar intracellular assimilation leads to the conclusion that the regulatory role of transport in different cases is manifested in a different degree. Thus, in cells of Ehrlich's ascitic carcinoma [6, 7], and probably in cells of Krebs-2 ascitic tumor [20] the permeability, evidently, does not limit the rate of glucose assimilation at all. At the same time, cells of Gardner's lymphosarcoma apparently resemble KI cells in the relationship between the rate of intracellular assimilation.

SUMMARY

Galactose competitively inhibited aerobic and anaerobic assimilation of glucose in intact cells of tissue cultures of monkey kidney cortex (Macacus rhesus). The inhibitory activity of galactose on glycolysis was not found in homogenate. This led to the conclusion that galactose apparently inhibited the transport of sugars through the cell sheath, reversibly blocking the active transport center. This carrier, apparently, is not hexokinase, since galactose has no effect on its activity.

Comparative determination of the relationship of concentration to the rate of glycolysis in intact cells and homogenates showed that saturation of the glycolytic system in intact cells occured with significantly larger concentrations of sugar, than saturation of the glycolytic system of homogenates. If one studies the considerable concentration gradient of glucose concentrations between the intracellular and extracellular fluids which we discovered earlier, the facts can be interpreted as an indication that the rate of glycolysis in KI cells (at least, at low glucose concentrations) is limited by the transport rate of this sugar across the cell membrane.

We wish to thank L. I. Romanova for her technical help in this work.

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EFFECTS OF SOME SUBSTRATES AND BIOLOGICALLY ACTIVE SUBSTANCES ON THE RATE OF RENEWAL OF PHOSPHORUS COMPOUNDS IN BRAIN SLICES

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There are indications in the literature that the rate of turnover of phosphorus compounds in the brain varies according to which substance is acting as the substrate for oxidation processes [1-3]. In vivo and in vitro studies of the renewal of phosphorus compounds have shown that the intensity of this process also depends on the functional state of the nervous system [4-6]. Numerous papers have been published, establishing the importance of the part played by the so-called biologically active substances acetylcholine (ACh), γ-aminobutyric acid (GAMA), and others in processes of nervous activity [7, 8]. However, the problem of the effects of oxidation substrates and of biologically active substances on the metabolism of phosphorus compounds in the brain has as yet been insufficiently studied.

The object of the present research was to study the intensity of metabolism of phosphoproteins (PP), phospholipids (PL), ribonucleic acids (RNA), and other phosphorus compounds in the presence of various oxidation substrates (glucose, fructose, L-glutamic acid), and of the biologically active substances ACh and GAMA. The experiments were with brain slices incubated with radioactive phosphorus.

METHODS

White rats were decapitated, and the brain was quickly removed, and washed in physiological saline; it was as far as possible dissected free of white matter. Slices of grey matter were cut with the aid of a razor, of a thickness of about 0.3 mm. As a general rule, slices from one hemisphere were taken for the experimental systems, with added substrates or ACh or GAMA, while those from the other hemisphere served for the control systems.

About 200 mg of brain slices were taken for each system. After 10 min of preincubation with the medium, the slices were incubated for 60 min at 37° in a Warburg apparatus, in a current of oxygen. The reaction vessels contained 3 ml of Krebs-Ringer-phosphate medium [9], to which we added labeled phosphate (5-20 μC of P^{32}). In those experiments in which we examined the effect of various substrates on the metabolism of phosphorus compounds glucose was replaced by the appropriate substrate, to a concentration of $1.3 \cdot 10^{-2}$ M; L-glutamic acid was added to its neutral salt. In other experiments, we added GAMA (10^{-2} M) or ACh (10^{-2} M) with eserine (10^{-4}M); in these experiments the substrate was glucose ($1.3 \cdot 10^{-2}$ M).

After completion of incubation, the slices were washed with 7-10 portions of ice-cold physiological saline, to remove contamination with radioactive phosphorus from the medium. They were then dried with filter paper, and frozen in liquid oxygen. The frozen slices were weighed on a torsion balance and ground up with cooled 10% TCA. The suspension was centrifuged, and the supernatant fluid was taken for determination of inorganic phosphate (P_{inorg}). The sediment was washed repeatedly with ice-cold 5% TCA and water, and lipids were extracted from the product (sequentially with acetone, ether, 3:1 alcohol – ether mixture, and 1:1 methanol – chloroform mixture). PP, RNA, and DNA were isolated by the method of Schmidt and Thannhauser, as modified by Vladimirov et al. [10]. For this purpose, the tissue residue was heated with 10% NaCl for 2 hr at 100°, to extract nucleic acids (NA). The NA were precipitated with lanthanum nitrate, and hydrolyzed with 0.3 N NaOH (18 hr at 37-38°). DNA was precipitated from the hydrolyzate by perchloric acid at pH 1, leaving RNA in solution. The PP remaining in the tissue residue was hydrolyzed with 1 N NaOH (20 hr at 37-38°).

The PL, RNA, and DNA fractions were wet-washed with $H_2SO_4 - HNO_3$ mixture.

Phosphorus was determined after extraction into isobutanol of the phosphomolybdic acid complex, by the method of Martin and Doty, as modified by Baranov [11]; we used a FEK-M photocolorimeter, with a red filter. Radioactivity was measured with the aid of an end-window counter, using B type equipment.

As an indicator of the rate of renewal of phosphorus compounds we took the relative specific activity (RSA), being the ratio of the specific activity (SA) of the given fraction to that of P_{inorg} of brain tissue.

TABLE 1. Respiratory Rate and Rate of Turnover of Phosphorus Compounds in Brain Slices. Incubation medium – Krebs – Ringer phosphate. Final concentration of substrate $1.3 \cdot 10^{-2}$ M. P^{32} – 20 μC per system. Incubation – 1 hr at 37°. SA expressed as cpm /μg P. RSA expressed as percentages of SA of P_{inorg}. The figures in parenthesis refer to the scatter of the results.

Substrate added	No. of expts.	O_2 uptake, μl O_2/100 mg in 60 min	SA and RSA of the phosphorus compounds			
			P_{inorg} (SA)	PP (RSA)	PL (RSA)	RNA (RSA)
Control (without substrate)	3	98 (91—102)	10444 (9030—11400)	2,5 (2,0—3,4)	0,15 (0,13—0,16)	0,10 (0,10—0,11)
Glucose	9	136 (120—186)	10713 (4389—137000)	13,5 (8,0—18,0)	0,25 (0,10—0,78)	0,22 (0,10—0,39)
Fructose	8	165 (137—191)	9899 (6870—11900)	9,5 (6,2—13,5)	0,26 (0,09—0,39)	0,20 (0,13—0,29)
L-glutamic acid	7	151 (130—170)	9491 (7540—12700)	3,8 (1,6—8,3)	0,13 (0,05—0,23)	0,10 (0,06—0,15)

The oxygen uptake of the slices was measured by Warburg's manometric method.

RESULTS

Renewal of phosphorus compounds in brain slices in the presence of different oxidation substrates. The basic source of energy for the brain is glucose, supplied under normal conditions from the blood stream [12]. Fructose is equally well metabolized by the brain, and can maintain ATP synthesis at a normal level, but it is more slowly glycolyzed than is glucose [13, 14]. The brain does not normally utilize L-glutamic acid for its energy requirements, although it can do so under conditions of glucose insufficiency [12, 15].

It has been shown that the rate of metabolism of phosphorus compounds is raised, when brain slices are incubated with glucose, whereas L-glutamic acid had no such action [1-3]. The literature references to the action of fructose are contradictory. A number of authors have shown that it has no effect, or only an insignificant one, on the rate at which the phosphorus compounds PP, PL, and RNA are metabolized [1, 2]. However, Schachner and others have found that incorporation of P^{32} is considerably increased in the PL fraction of brain slices incubated with fructose [3].

Our findings regarding the metabolism of phosphorus compounds in the presence of different substrates, presented in Table 1, show that incorporation of P^{32} takes place in the absence of added substrates, although at a very slow rate.

Special experiments showed that the SA of the phosphorus fractions of brain slices was about 15 times greater when the systems were incubated than without incubation. It may be concluded that the radioactivity of the phosphorus compounds of incubated systems cannot have been due to contamination with P^{32} from the medium.

The metabolic rate of the phosphorus compounds is greatly intensified when glucose is present in the medium. The rate of turnover of PL and RNA was increased to the same extent by fructose as by glucose, but of PP to a somewhat smaller extent. These findings are not in agreement with published reports that addition of fructose does not intensify the metabolism of phosphorus compounds [1, 2]. The reason for these divergent findings is not clear. It may possibly be related to our use of a more adequate medium for incubation than was used by other authors.

The rate of renewal of the phosphorus compounds was the same as in the absence of a substrate when L-glutamic acid was added instead of glucose or fructose; this is in accordance with the findings of other authors [1, 2].

The SA of P_{inorg} found with different substrates was the same in all cases (Table 1). This indicates that the given substrates did not affect the rate of penetration of P^{32} into the brain cells. The rate of uptake of oxygen by the slices is practically the same with glucose, fructose, or L-glutamic acid as the oxidation substrate (Table 1). It follows that increased oxygen uptake is not necessarily associated with increase in the rate of renewal of the phosphorus compounds examined by us.

Effect of ACh on the metabolism of phosphorus compounds in brain slices. ACh is known to function as a chemical transmitter of excitation at synapses of the nervous system. It has a stimulating action on the cerebral cortex [7]. ACh formation is closely bound up with processes of cell metabolism [16]. There are only scanty data in the literature on the effect of ACh on the metabolism of the P compounds PP, NA, and PL. The investigation of this problem is for this reason of undoubted interest.

TABLE 2. Effect of ACh on the Rate of Renewal of Phosphorus Compounds in Brain Slices. Final concentration of ACh – 10^{-2} M. P^{32} – 5-10 μC per system. Other explanations as in Table 1.

Compound	No. of expts.	Control expts.	Expts. with added ACh	Change in RSA, in relation to the control, %	Value of P
P_{inorg} (SA)	35	2709	2689	—	—
PP (RSA)	28	9,0 ±0,7	11,5 ±0,9	+28	<0,05
PL (RSA)	26	0,19±0,02	0,27±0,03	+42	<0,05
RNA (RSA)	20	0,38±0,05	0,26±0,03	—31	<0,05

During excitation, the ACh of isolated parts of the brain may achieve higher concentrations than those reported in the literature for the whole brain [4]. On these grounds we applied much higher concentrations of ACh in our experiments than those present in brain tissue as a whole. It is known that only 50% of the ACh present in 0.1 M solutions undergoes spontaneous decomposition over 41 days of incubation at 37.5° [17]. We could therefore assume that under our conditions, with a final ACh concentration of 10^{-2} M, any considerable decomposition of ACh could be excluded over the incubation times applied.

We found awide scattering of the numerical results of individual experiments, for which reason we increased the number of experiments and subjected the results to statistical evaluation [18].

It is evident from the results presented in Table 2 that, at ACh concentrations of 10^{-2} M, the rate of renewal of PP and PL rises, and of RNA falls. This difference is statistically significant. The SA of DNA could not be determined, because of the low activity of the fractions.

Experiments in which the slices were incubated with eserine (10^{-4} M), without ACh, showed that eserine alone had no effect on the rate of renewal of the specified compounds.

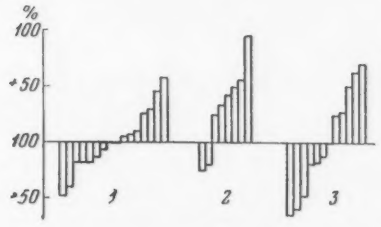
The constancy of the SA of P_{inorg} can be taken as evidence that ACh does not affect the rate of penetration of phosphorus into the cells. We found that addition of ACh did not affect the contents of PP, PL, RNA, and DNA phosphorus in brain slices.

We were unable to observe any changes in the metabolic rate of the contents of these phosphorus compounds following incubation with lower concentrations of ACh (10^{-3} M, 10^{-4} M). Some tendency towards increase in the rate of renewal of phosphorus at these lower ACh concentrations was observed for PL only (see figure).

Reports that ACh, at concentrations of 10^{-3} M and 10^{-4} M, causes a rise in the rate of metabolism of PL in brain slices or homogenates have appeared in the literature [19, 20]. We have not, however, seen any papers which included a statistical evaluation of the results of a large number of experiments, or in which the RSA was used as a measure of the rate of renewal of PL, as has been done in the present paper.

It is of interest that in the presence of 10^{-2} M ACh the rates of renewal of PP and PL rose, while that of RNA fell. The reason for this effect is not clear.

Effect of GAMA on the rate of renewal of phosphorus compounds in brain slices. Numerous investigators have recently directed their attention to the study of the role of GAMA in nervous tissues. GAMA has been reported to be a normal metabolic product, and to be one of the substrates of oxidation in brain [21, 22]; on the other hand, it has been thought to function as a factor inhibiting synaptic transmission [8, 23]. Elucidation of the way in which GAMA affects the metabolism of the nervous system would be of great importance in furthering our understanding of the mechanism of nervous processes. For this reason we undertook the study of its effect on the rate of renewal of the phosphorus compounds PP, PL, and RNA, and also of lipoproteins (LP). The phosphorus-containing component of LP was extracted for 2 hr at room temperature with an acidified



Effect of acetylcholine (10^{-3} M, 10^{-4} M) on the rate of renewal of phosphoproteins, phospholipids, and RNA in brain slices. The columns represent change in the RSA of phosphorus compounds, as % of the control values. 1) Phosphoproteins; 2) phospholipids; 3) RNA. Each column applies to a separate experiment, for which the control is taken as 100%.

TABLE 3. Rate of Renewal of Phosphorus Compounds in Brain Slices in the Presence of GAMA. Final concentrations of GAMA – 10^{-2} M; P^{32} – 5-10 μC per system. Other explanations as in Table 1.

Experimental conditions	P_{inorg} (SA)	PP (RSA)	PL (RSA)	RNA (RSA)	LP (RSA)
	Number of experiments				
	18	13	10	13	10
Control	2891 (2070—4700)	8,4 (4,3—11,2)	0,32 (0,12—0,67)	0,47 (0,05—1,37)	5,7 (3,4—9,2)
With addition of GAMA	2843 (2130—5026)	7,8 (5,0—13,5)	0,29 (0,13—0,80)	0,46 (0,15—1,53)	5,6 (3,4—8,7)

TABLE 4. Rate of Renewal of Phosphorus Compounds in Brain Slices in the Presence of GAMA at Concentrations of 10^{-3} M and 10^{-1} M. Explanation of Table as for Table 3.

Experimental conditions	P_{inorg} (SA)	PP (RSA)	PL (RSA)	RNA (RSA)	LP (RSA)
Control	2380, 2070	10,0, —	0,67, 0,57	0,60, 0,53	9,0, 9,2
With addition of GAMA (10^{-3} M)	2220, 2160	11,0, 14,4	0,57, 0,40	0,34, 0,70	7,8, 7,2
Control	3420, 4635	7,2, 6,3	0,21, 0,24	0,11, 0,05	3,4, 5,3
With addition of GAMA (10^{-1} M)	3250, 4320	5,7, 5,5	0,22, 0,25	0,11, 0,18	3,2, 2,4

chloroform – methanol mixture (HCl was added to a final concentration of 0.04 N), as recommended by Baranov [23].

We found that at a concentration of 10^{-2} M GAMA had no effect on the rate of renewal of the specified compounds (Table 3).

The same result was obtained with GAMA concentration of 10^{-3} M and 10^{-1} M (Table 4).

It is known from the literature that, at the concentration used in our experiments, GAMA exerts a definite physiological effect, viz., inhibition of nerve transmission, and modification of the nature of electric potentials [24]. In vitro experiments have shown that, in the presence of glucose, brain slices are freely permeable to GAMA [25]. The absence of any effect of GAMA on the rate of renewal of phosphorus compounds cannot therefore be ascribed to its slow penetration into the slices. It appears that GAMA affects the functioning of the nervous system in a way which does not affect the rates of renewal of the phosphorus compounds examined by us.

SUMMARY

Both the rate of oxygen uptake and the rates of renewal of phosphorus, phospholipids, and ribonucleic acid in rat brain slices are raised when glucose or fructose are added to the incubation medium.

Although addition of L-glutamic acid raises the rate of uptake of oxygen, it does not affect the rates of renewal of the phosphorus of phosphoproteins, phospholipids, and RNA.

Acetylcholine at a concentration of 10^{-2} M raises the rate of turnover of the phosphorus of phosphoproteins and phospholipids, but lowers it for RNA, when incubated with rat brain slices. γ-Aminobutyric acid has no effect on the metabolism of phosphoproteins, phospholids, RNA, and lipoproteins of brain slices.

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STABILIZING EFFECT OF FLAVONOIDS ON L-ASCORBIC ACID

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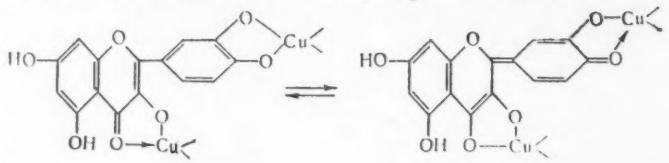
A number of papers have been published on the mutual relations between ascorbic acid and flavonoid substances, mostly of the "vitamin P type"; the problem has been usually approached from a physiological angle. All the authors are in agreement that substances of the type of vitamin P reinforce the action of ascorbic acid. Natural mixtures of ascorbic acid with flavonoid substances not only protect against scurvy, but also stimulate growth, and raise the ascorbic acid content of various organs, in particular of the adrenals, spleen, kidneys, etc.; the flavonoids thus act not only as growth factors, but also as protective and stabilizing factors.

The mutual relations between ascrobic acid and flavonoid substances have been insufficiently studied from a chemical point of view. Only a small number of researches have been devoted to this subject, and the findings have in some cases been contradictory.

A number of authors have reported that ascrobic acid is much more stable in natural juices than when in pure solutions. Shamrai [1] ascribed the protective action of such juices to the presence in them of tannin and gallic acid, and he postulated that these substances react chemically with ascorbic acid, and that the reaction products possess higher stability than does ascorbic acid. Kardo-Sysoeva and Nissenbaum [2] reported the presence in tomatoes of a thermostable factor, which protected ascorbic acid from oxidation during boiling; the stabilizer inhibits the catalytic action of copper. Parrot and Cotereau [3, 4] found that catechols, when present at certain concentrations, also have a stabilizing effect on ascorbic acid. Analogous results were reported by Gego [5] and by Tria and Barnabei [6], who found that rutin had a stabilizing effect on ascorbic acid at pH 8, whereas in acid solutions it catalyzed its oxidation. Novotelnov et al. [7], have made a detailed study of the effect of the flavonoid fraction of rose-hips on ascorbic acid. They found that the stabilizing effect was due to polyphenol complexes, notably to substances of the type of rutin. The flavonoids of rose-hips lower the concentration of dissolved oxygen in the solutions, in this way protecting ascorbic acid from oxidation.

Numerous authors have investigated the relationships between ascrobic acid, flavonoids, and heavy metals. Masguelier [8] examined the effect of substances of the type of vitamin P on the stability of ascorbic acid in the presence of Cu^{++}. He found that leucoanthocyanin inhibited oxidation of ascorbic acid, rutin had practically no effect on the process, and esculin catalyzed destruction of ascorbic acid. Only leucoanthocyanin inhibited oxidation of ascorbic acid in the presence of ascorbic dehydrogenase, whereas rutin and esculin had no effect on this reaction.

Kelley and Wattsova [10, 11] showed that inhibition of oxidation of ascorbic acid was found only in compounds which possessed a functional group capable of complexing heavy metals. According to Kelley and Wattsova, complexing of copper by flavonoids takes place according to the scheme:



It is clear from this review of the literature that the protective effects of flavonoids on ascorbic acid has not yet been definitively elucidated. The available data do not permit of the definite acceptance or rejection of any of the current theories and hypotheses, some of the propositions of which are mutually contradictory.

The present paper is devoted to a study of the kinetics of destruction and stabilization of ascorbic acid in the presence of flavonoid substances, and in pure solutions, both in the presence and in the absence of heavy metals catalyzing oxidation.

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EXPERIMENTAL

Apparatus. For the investigation of the kinetics of oxidation of ascorbic acid (AA) we used a Heyrovsky B-301 polarograph (Zbrojevka Factory), with a Kalusek cell and a saturated mercury-sulfate electrode. We used a capillary tube which, at a height of the reservoir h = = 65 cm, gave a flow rate of 2.9 mg/sec, with a dropping interval of 3.1 sec.

Solutions. Standard AA solution, 10^{-3} M, in redistilled water; $5 \cdot 10^{-3}$ M rutin (purchased from Lachema) in methanol; $5 \cdot 10^{-3}$ M naringin (from Sunkist Growers) in ethanol; $5 \cdot 10^{-3}$ M quercitrin (from Geigy) in methanol; 0.5% solution of orange flavonoids in redistilled water; methanol and ethanol (AR), further purified by treatment with silver nitrate and aluminum amalgam; acetate buffers, prepared from analytical reagents dissolved in redistilled water.

Oxidation of AA took place in a thermostatted polarographic cell, and readings were taken continuously or at 5-min intervals. The buffer solution was in a thermostatted Kalusek cell, and the AA solution in a special cell, also thermostattted, and connected with the Kalusek cell (Fig. 1). After the AA solution had reached the desired temperature it was transferred to the buffer solution; destruction of AA was followed by means of the following procedure.

The acetate buffer and the other components of the system (previously tested in special experiments) were pipetted into the Kalusek cell, and the AA solution into a separate cell. Air was bubbled through concentrated sulfuric acid, and then through the solutions in both cells, at a rate of about 300 ml/min. This assures thorough mixing of the solutions, and at the same time keeps them saturated with oxygen. After thermal equilibration (about 7 min), the AA solution was run into the buffer solution, and air was passed for a further 2 min, after which a potential was applied to the electrode, such as would give the optimum conditions for reading the limiting current for AA. The limiting current was recorded continuously, or at 5-min intervals. The height of the AA wave was derived, subtracting from it the value found for the control (the control system differed from the experimental one in that an equal volume of redistilled water was run into the polarographic cell instead of AA solution).

Interpretation of the polarograms obtained. The height of the AA wave was determined as described above, and curves relating wave heights to time were drawn. The curves of Fig. 2a show that the rate of destruction of ascorbic acid falls with diminishing concentration. We found that the logarithm of the concentration of AA varied linearly with time, and that the kinetics corresponds with those of a first order reaction. The rate constants were derived from the equation:

$$K = \frac{2.303}{t} \cdot \log \frac{h_0}{h_0 - h}, \text{ where}$$

K is the velocity constant, t is time in minutes, h_0 is the AA concentration at time t_0, and h is the fall in AA content over time t.

The stabilizing effect of flavonoids on AA was examined both in the absence and the presence of some heavy metals.

Stabilizing effect of flavonoids on ascorbic acid in the absence of heavy metals. Before examining the effects of flavonoids we investigated the breakdown of ascrobic acid in buffer solutions. We found that, notwithstanding rigorous standardization of the experimen-

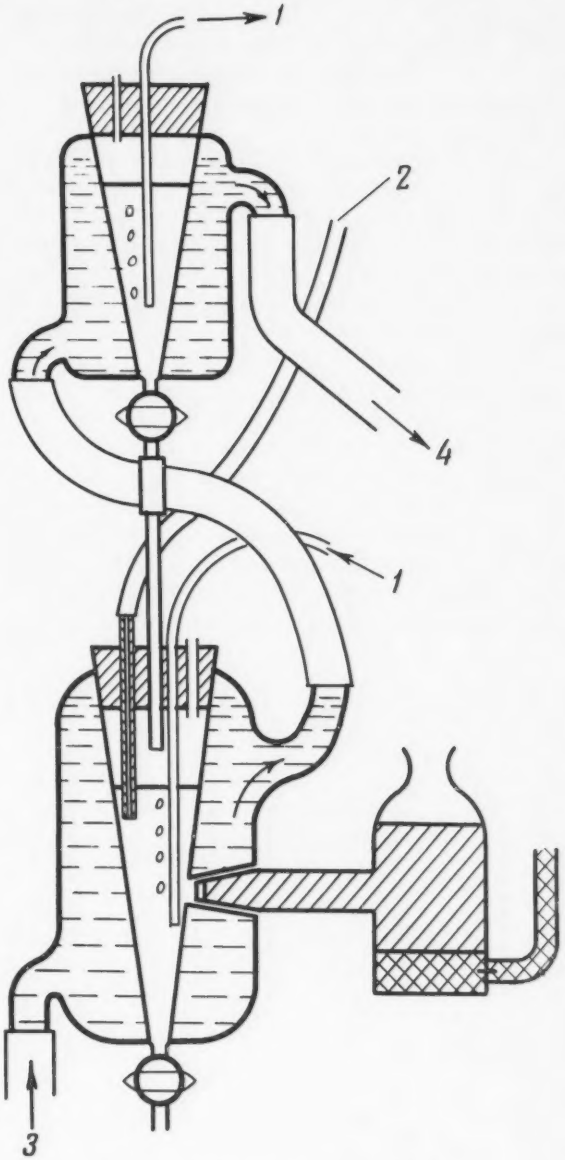

Fig. 1. Additional equipment for preparing samples for polarography. 1) Air; 2) mercury reservoir; 3) influx; 4) efflux.

tal conditions, the rate of destruction of ascorbic acid varied from experiment to experiment. There was accordingly a wide scatter of the results obtained for the rate constants in different experiments. For the stabilization of AA we used orange flavonoids, at a concentration of 0.13%.

We placed 2.5 ml of 0.2 M acetate buffer, 4 ml of an aqueous 0.5% solution of orange flavonoids, and 3.5 ml of redistilled water into a thermostatted Kalusek polarography cell. Into a second cell we placed 2.5 ml of 10^{-3} M ascorbic acid and 2.5 ml of redistilled water. The two solutions were mixed after air had been bubbled through them for 7 min, and passage of air was continued for a further 2 min, after which the limiting current was recorded at 5-min intervals, beginning from the moment of mixing.

The differences shown in Table 1 lie within the range of the experimental error, and do not constitute evidence of the stabilizing action of orange flavonoids. It may be concluded from these results that this mixture of flavonoids has no direct stabilizing action on ascorbic acid. Similar results were obtained with pure rutin, quercitrin, and naringin.

Stabilizing effect of flavonoids on ascorbic acid in the presence of heavy metals. It is known from the literature that heavy metals catalyze the breakdown of ascorbic acid. We therefore examined the stabilization of AA in the presence of heavy metals.

Our experiments were performed with copper, which is a typical catalyst of AA decomposition. We first examined the kinetics of the reaction when Cu^{++} was present in the buffer solution. The reaction was found to conform to first order reaction kinetics. During the reaction copper oxidized AA to hydroascorbic acid, being itself reduced to the cuprous form, which is reoxidized to Cu^{++} by atmospheric oxygen. This process is then repeated until the AA has all been oxidized.

The following emerged from the experiments in which the kinetics of oxidation of AA in the presence of Cu ions were studied.

The rate constant of oxidation of AA rose parallel with rise in pH, to about 7, and showed a linear dependence on the Cu^{++} concentration, from $0.66 \cdot 10^{-6}$ M up. At Cu^{++} concentrations lower than $0.66 \cdot 10^{-6}$ M the velocity constants found deviated from the theoretical values. The reaction rate falls in such solutions, and as the Cu^{++} concentrations were further diminished the values of the velocity constants fell progressively to that found in the absence of metals (Fig. 2).

The rate of oxidation of ascorbic acid rose exponentially with rising temperature (Fig. 2b). The velocity of the reaction of oxidation of ascorbic acid in the presence of Cu^{++} does not depend on the ionic strength. The deviations recorded lie within the limits of experimental error.

The rate of oxidation of AA in a partly non-aqueous medium (13% ethanol or methanol) was about 20% lower than in the usual aqueous medium; this effect may be related to the lower solubility of oxygen in the aqueous alcoholic medium.

In our next series of experiments we studied the stabilizing effects of flavonoids. We examined the effect of adding pure flavonoids (rutin, quercitrin, naringin) and of natural mixtures of orange flavonoids, as well as of black currant juice.

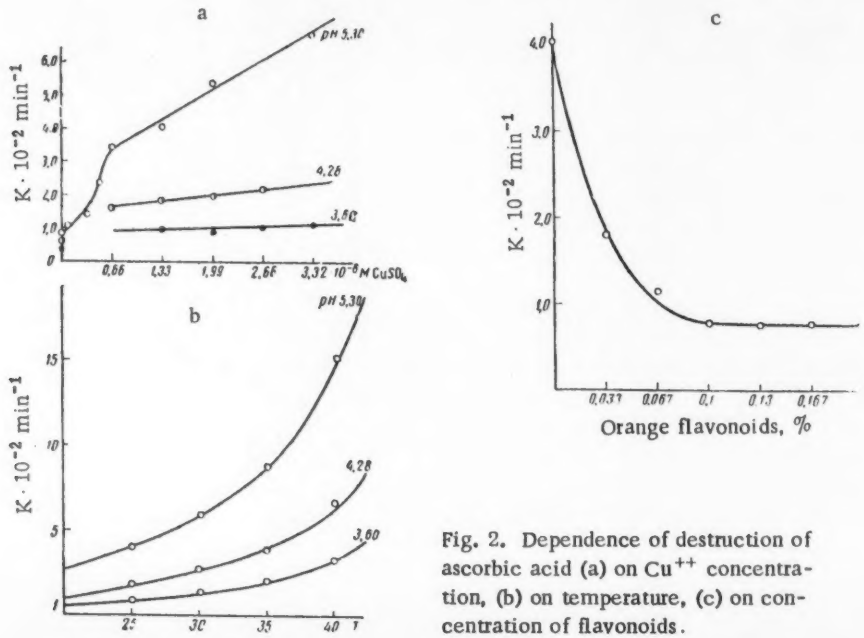

Fig. 2. Dependence of destruction of ascorbic acid (a) on Cu^{++} concentration, (b) on temperature, (c) on concentration of flavonoids.

TABLE 1. Effect of pH on the Stabilizing Action of Flavonoids Pure Solutions of AA Used

pH	Concentration		$K \cdot 10^{-2}$ min^{-1}	
	AA, M	orange flavonoids, %	without flavonoids	with flavonoids
3,60 4,28 5,30	$1,66 \cdot 10^{-4}$	0,13	0,30—0,41 0,54—0,67 0,50—0,90	0,32—0,40 0,50—0,60 0,35—0,56

TABLE 2. Effect of pH on the Stabilizing Action of Pure Flavonoids in the Presence of Cu Ions

pH	Concentration		Without flavonoids, $K \cdot 10^{-2} m^{-1}$	Conc. of flavonoids, M	With flavonoids, $K \cdot 10^{-2}$ min^{-1}	Stabilizing effect, %
	AA, M	$CuSO_4$, M				
			Rutin			
3,6 4,28 5,30	$1,66 \cdot 10^{-4}$	$1,33 \cdot 10^{-6}$	1,14 2,37 3,34	$8,3 \cdot 10^{-4}$	0,60 1,09 1,43	47,4 53,0 57,2
			Quercitrin			
3,60 4,28 5,30	$1,66 \cdot 10^{-4}$	$1,33 \cdot 10^{-6}$	1,14 2,37 3,34	$8,3 \cdot 10^{-4}$	0,72 1,38 1,96	36,7 41,0 41,3
			Naringin			
3,60 4,28 5,30	$1,66 \cdot 10^{-4}$	$1,33 \cdot 10^{-6}$	0,88 1,69 3,58	$8,3 \cdot 10^{-4}$	0,82 1,34 2,40	6,8 20,6

TABLE 3. Effect of Temperature on the Stabilizing Action of Pure Flavonoids in the Presence of Cu Ions

r	Concentration			pH 3.60			pH 4.28			pH 5.30		
				$K \cdot 10^{-2} min^{-1}$			$K \cdot 10^{-2} min^{-1}$			$K \cdot 10^{-2} min^{-1}$		
	AA, M	$CuSO_4$, M	Flavonoids, M	without flavonoid	with flavonoid	Stabilizing effect, %	without flavonoid	with flavonoid	Stabilizing effect, %	without flavonoid	with flavonoid	Stabilizing effect, %
						Rutin						
25 30 35 40	$1,66 \cdot 10^{-4}$	$1,33 \cdot 10^{-6}$	$8,3 \cdot 10^{-4}$	1,14 1,58 2,10 2,69	0,60 0,88 1,78 2,07	47,4 44,3 15,2 22,4	2,34 2,79 3,85 5,29	1,09 1,74 2,27 3,30	53,4 37,6 41,0 37,6	3,34 5,16 6,99 3,46	1,43 2,05 2,94 9,14	57,0 60,2 57,8 62,4
						Quercitrin						
25 30 35 40	$1,66 \cdot 10^{-4}$	$1,33 \cdot 10^{-6}$	$8,3 \cdot 10^{-4}$	1,14 1,58 2,10 2,69	0,72 1,09 1,59 2,79	36,7 31,0 24,2 Ivv.	2,34 2,79 3,85 5,29	1,38 1,82 2,98 4,48	41,0 34,6 22,5 15,2	3,34 5,16 6,99 9,14	1,96 2,70 3,76 4,57	41,3 47,7 37,3 50,0
						Naringin						
25 30 35 40	$1,66 \cdot 10^{-4}$	$1,33 \cdot 10^{-6}$	$8,3 \cdot 10^{-4}$	0,88 1,14 1,84 3,01	0,82 0,92 1,86 2,71	6,8 19,2 Ivv. 10,0	1,69 2,66 3,37 4,46	1,34 2,12 2,98 4,45	20,6 20,2 11,5	3,58 4,07 5,08 8,29	2,40 4,04 5,11	33,0 20,3 38,2

We placed 2.5 ml of 0.2 M acetate buffer, 2.0 ml of 10^{-5} M $CuSO_4$ (final concentration in the system– $1.33 \cdot 10^{-6}$ M), a solution of the flavonoid under investigation, and redistilled water to 10 ml, into a Kalusek cell. Into a second cell we introduced 2.5 ml of 10^{-3} M AA and 2.5 ml of redistilled water. The experiments were conducted as for systems with flavonoids, but without Cu^{++}.

We found that the stabilizing effect of pure flavonoids increased with rising pH of the solutions. The stabilizing effect of the flavonoids varies in the descending order: rutin, quercetrin, naringin. At pH 5.30 and 25° rutin lowered the oxidation rate by 57%, quercitrin by 41.3%, and naringin by 33% (Table 2).

At low pH values, the stabilizing effect of the flavonoids fell with rising temperature. The effect of temperature on the rate of oxidation of AA was less considerable at higher pH. Thus, at pH 5.30 the stabilizing effect at 40° was practically identical with that at 25°. The best results were obtained with rutin and quercitrin, the stabilizing effect of naringin being much smaller (Table 3).

We also examined the effects of pH, flavonoid concentration, and temperature on the stabilizing action of a mixture of orange flavonoids.

The following results were obtained for the pH dependence of the stabilizing effect:

The stabilizing effect of the flavonoids, under the above-specified conditions, is so great as practically to abolish the catalytic effect of copper ions (Table 4). We have not compared these results with those found in experiments on ascorbic acid solutions not containing copper ions, at raised temperatures, in view of the wide scatter of the results obtained in the latter case, rendering the experimental error much greater than with stabilizers.

Very similar results were obtained from experiments with black currant juice, which suppressed the catalytic effect of copper ions in up to 15-fold dilution. It is understandable that other components, such as sulfhydryl compounds, pectins, sugars, etc., present in biological material, can affect the rate of oxidation of ascorbic acid. The experiments with pure flavonoids leave no doubt, however, that they are able to exert a stabilizing effect on ascorbic acid.

DISCUSSION OF RESULTS

In this research we examined the rates of oxidation of ascorbic acid in the presence and absence of flavonoids, and in the presence and absence of copper ions. Our findings showed that the falavonoid substances taken had no stabilizing effect in the reaction proceeding in the absence of copper, but that they were good inhibitors of the copper-catalyzed reaction. Of the pure flavonoids

pH	Concentration AA, M	Concentration $CuSO_4$, M	$K \cdot 10^{-2}$ min^{-1} without flavonoids	Flavonoids, %	$K \cdot 10^{-2}$ min^{-1} with flavonoids	Stabilizing effect, %
3.60	$1.66 \cdot 10^{-4}$	$1.33 \cdot 10^{-6}$	0.97	0.13	0.41	54
4.28			1.77		0.90	49.2
5.30			4.05		0.75	81.5

Comparing these results with those found in the absence of copper ions, it appears that the stabilizing effect is so great, particularly at high pH values, that the catalytic action of Cu ions has practically been abolished.

The dependence of the stabilizing effect of flavonoids on their concentration was studied in a series of experiments. For this purpose we used only the mixture of orange flavonoids, since it was only with this mixture that we could achieve total suppression of the catalytic action of Cu ions. We found that the value of the rate constant of the reaction fell with rising concentration of flavonoid, and that the stabilizing effect rose (Fig. 2c). The catalytic action of copper was totally abolished at a flavonoid concentration of 0.1%, the value of the velocity constant becoming the same as for solutions not containing copper. The rate of reaction was not affected by further increase in flavonoid concentration.

studied, rutin and quercitrin gave the greatest stabilizing effect, amounting to about 50% under the given experimental conditions. The flavonol naringin was less effective in this respect. This difference might be ascribed to the presence in the side-ring of only one hydroxyl, at position 3', whereas the other flavonols have two hydroxyls, at positions 3' and 4', which are capable of complexing heavy metals. Very high protective effects were shown by a natural mixture of orange flavonoids and by black currant juice. At a concentration of 0.1%, the orange flavonoids totally abolished the catalytic action of copper ions present at a concentration of about 10^{-6} M. Black currant juice was equally effective in 15-fold dilution. These findings suggest that it would be advisable to stabilize the AA present in some fruit juices by adding juices of a high flavonoid content (orange, lemon, black currant, rose hips).

TABLE 4. Effect of Temperature on the Stabilizing Action of Orange Flavonoids in the Presence of Cu Ions

T	Concentration			pH 3.60			pH 4.28			pH 5.30		
	AA, M	$CuSO_4$, M	orange flavonoids, %	$K \cdot 10^{-2} min^{-1}$			$K \cdot 10^{-2} min^{-1}$			$K \cdot 10^{-2} min^{-1}$		
				without flavonoid	with flavonoid	Stabilizing effect, %	without flavonoid	with flavonoid	Stabilizing effect, %	without flavonoid	with flavonoid	Stabilizing effect, %
25	$1,66 \cdot 10^{-4}$	$1,33 \cdot 10^{-6}$	0,13	0,97	0,46	52,6	1,86	0,90	51,6	4,05	0,75	81,5
30				1,43	0,78	45,5	2,78	1,43	48,5	6,03		
35				2,01	1,07	46,8	3,88	1,74	55,1	8,88	1,19	86,5
40				3,37	1,47	56,4	6,87	2,36	65,6	15,30	1,66	89,2

Our experiments confirmed that the stabilizing effect of flavonoids on ascorbic acid is not due to interference with oxidation - reduction processes, but is exerted indirectly. Flavonoids, which have marked complexing properties, readily bind heavy metals present in solutions, to yield stable complexes, thereby abolishing their catalytic action.

SUMMARY

Flavonoid substances have no stabilizing effect on ascorbic acid in solutions not containing catalytically active ions, but they inhibit oxidation due to the presence of copper ions.

The protective action of some pure flavonoids varies in the diminishing order; rutin, quercetrin, naringin.

Very high stabilizing effects were given by a natural mixture of orange flavonoids and by black currant juice.

The protective effect of flavonoids may be ascribed to their ability to form stable complexes with heavy metal ions, thereby abolishing their catalytic action on oxidation of ascorbic acid.

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SOME PROPERTIES OF PURIFIED GLUTAMINE TRANSAMINASE*

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Since the discovery in 1947 by Greenstein and Carter [1] of an enzyme system in animal tissues which catalyzed deamidation of glutamine in the presence of keto acids ("glutaminase II"), numerous papers have been published, dealing with the reaction mechanism, and with a number of the properties of this system [2-8]. These authors worked, however, with crude enzyme preparations, and enzymatic activity was assessed either from the amount of ammonia formed, or from the fall in the content of glutamine amino groups. The data obtained by these workers thus related to the processes of coupled transamination and of deamidation of L-glutamine, and represented the sum of the effects of two different enzymes -- Glu-NH_2 transaminase and ketoamidase [8]. The preparation of purified Glu-NH_2 transaminase, free of glutaminase and of ketoamidase, has been described by us earlier [9, 10]. We have, using such a preparation, examined the electrophoretic mobility of Glu-NH_2 transaminase [9, 10], and its action on the optical isomers of cycloserine [11]. The present paper reports the results of the study of certain other properties of Glu-NH_2 transaminase.

EXPERIMENTAL

We used lyophilized preparations of Glu-NH_2 transaminase, previously purified by electrophoresis in vertical columns packed with cellulose powder [9, 10].

The activity of the enzyme was assayed as follows: the enzyme solutions contained 0.1-0.5 mg of Glu-NH_2 transaminase protein in 1-2 ml. Since glutaminase and ketoamidase were absent from our preparation, its activity could be assessed from the amount of newly formed amino acid, by the transamination reaction; the amount of amino acid was determined spectrophotometrically, from the optical density of solutions of the copper–ninhydrin complex eluted from paper chromatograms [12, 13].

EXPERIMENTAL RESULTS AND THEIR DISCUSSION

Reaction kinetics. The curve of Fig. 1 shows that the amount of phenylalanine formed rises regularly with time of incubation of the system Glu-NH_2–PhP–enzyme; over the first 90 min the rate of Phe formation is close to linear.

pH-Optimum of Glu-NH_2 transaminase. The pH optimum for deamidation of Glu-NH_2 in the presence of pyruvate has been reported by Greenstein [2] as being at pH 7.2, and at 7.7 by Errera [15]; Meister [8] found that the optimum with phenylpyruvate was at pH 8.5. With α-ketoisocaproate the optimum pH was found by Greenstein to be 8.8. These data were derived from experiments in which crude enzyme preparations containing glutaminase [2] and ketoamidase [6-8] were used. The glutamine deamidating activity in the presence of α-keto acids was derived by these authors from the amounts of ammonia formed, or from the fall in Glu-NH_2 content of the systems; the results therefore represent the effects of the summation of the action of two (or three) enzymes, viz., Glu-NH_2 transaminase, ketoamidase (and glutaminase).

We determined the pH optimum of purified Glu-NH_2 transaminase with various substrates (PhP, pyruvate, and α-ketoisocaproate), using citrate (0.05 M; pH 4.3-5.5), phosphate (0.05 M; pH 5.9-7.4), veronal (0.05 M; pH 7.2-9.2), borate (0.025 M; pH 9.3-9.9);, and carbonate (0.05 M; pH 9.2-10.7) buffer solutions. The pH of the buffer solutions was verified by measurement with a glass electrode, using a LP-5 potentiometer. The results of the experiments are represented graphically in Fig. 2. It is evident from the curves of Fig. 2 that, with PhP as the acceptor of the NH_2 group, the pH optimum of Glu-NH_2 transaminase is at 9.1, with pyruvate at 8.2, and with α-ketocaproate at 8.7. We could find no appreciable differences in the activity of the enzyme in the different buffer solutions in zones in which the pH values overlapped.

The optimum pH found by us for purified L-glutamine transaminase differ from those reported by the above authors for the combined processes of transamination and deamidation; only in the experiments with α-ketoisocaproate did the results coincide.

*Explanation of abbreviations: Glu-NH_2 transaminase - glutamine transaminase; Glu-NH_2 - glutamine; PhP - phenylpyruvate; Phe - phenylalanine; Ala - alanine; K_s - Michaelis constant; PCMB - p-chloromercuribenzoate; IAA - iodoacetamide; GSH - reduced glutathione; GSSG - oxidized glutathione; VB - veronal buffer.

The pH values of the most alkaline borate and carbonate buffer solutions, of pH > 9.7, are probably higher than the true values, since the glass electrode does not give reliable results for solutions of such alkalinity. The very considerable activity of glutamine transaminase in reactions involving PhP and α-isocaproate (but not pyruvate), at pH > 10, is nevertheless noteworthy. Control experiments in which the substrates were incubated without enzyme in the same buffer solutions showed that non-enzymic transfer of NH_2-groups did not take place.

Thermal inactivation of glutamine transaminase. Crude glutamine transaminase is known to be relatively thermostable, for which reason heat treatment of short duration may be applied for the denaturation of ballast proteins in the purification of this enzyme [9, 10]. We examined the thermostability of purified glutamine transaminase, over a range of temperatures. A solution of purified enzyme (0.25 mg/ml) was heated at various temperatures, from 40 to 80°, for 5 min. The solutions were then cooled rapidly to room temperature, substrate was added, and the systems were incubated at 37°. The activity of glutamine transaminase was unaffected by heating at 50°, while it was 65% inactivated by heating at 80°. The effects of these and of intermediate temperatures are shown graphically in Fig. 3. Even heating for 2 min in a boiling water bath followed by incubation at 37° for 1 hr with PhP in borate buffer of pH 9.8, still left considerable residual activity (36% of that of unheated enzyme in a control system).

Temperature dependence of the reaction of transamination of glutamine. The effect of temperature on the rate of transamination of glutamine (14 μmoles) with PhP (14 μmoles) was examined (1 hr incubation).

The curve of Fig. 4 shows that the rate of the enzymic reaction was tripled over the temperature interval 15-45°.

Determination of the dissociation constant of the glutamine – enzyme complex. For the determination of the apparent dissociation constant (K_s; Michaelis constant) of the intermediate glutamine – glutamine transaminase complex we measured the rates of transamination in systems in which the concentration of amino group acceptor was kept constant ($7 \cdot 10^{-3}$ M), while that of glutamine was varied, from 3.5 to $28 \cdot 10^{-3}$ M. The total volume of the systems was 1 ml, and the incubation time was 1 hr, at 37°. A Lineweaver–Burk plot [14] was made of the ΔPhe values found, relating the reciprocal of the velocity (1/V) to that of the glutamine concentration (1/S). The value of K_s derived from this plot (Fig. 5) amounted to $3.33 \cdot 10^{-3}$ M. Our results confirm the observations of Greenstein [2, 15] and Meister [16], that above certain limits increase in concentration of the α-keto acids lowers the activity of glutamine transaminase. With PhP inhibition of enzyme activity begins to appear when its concentration exceeds $7 \cdot 10^{-3}$ M. It was not, therefore, possible to achieve an accurate deriviation of the value of K_s for systems containing α-keto acids.

Denaturation of glutamine transaminase by urea. We could find no references in the literature to the quantitative aspects of inhibition of transaminases by the action of denaturing agents such as urea, which affect the tertiary structure of the enzyme protein molecule. We found that urea inhibits glutamine transaminase activity. The enzyme solution was preincubated with urea for 10 min at 25°, substrate was added, and the system was incubated for 1 hr at 37°. The results of these experiments are presented in Fig. 6, from which it appears that 21% inhibition is caused by 3 M urea, while 5 M urea gives 68% inhibition. Inactivation of glutamine transaminase by urea was found to be irreversible. Reactivation of enzyme preincubated with urea could not be achieved by dialysis against several changes of distilled water, with stirring, for 20 hr.

Effect of sulfhydryl poisons and reactivators on glutamine transaminase activity. Sporadic references are made by the authors cited above to the effects of a number of substances (e.g., cyanide, NaF, azide, cysteine,

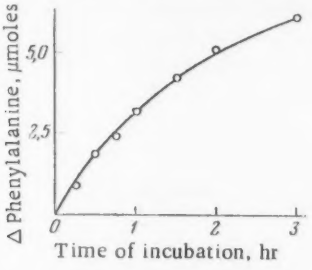

Fig. 1. Time - activity curve of the reaction. The systems contained 14 μmoles each of Glu-NH_2 and PhP in 2 ml of 0.05 M VB, pH 8.4, and were incubated at 37°.

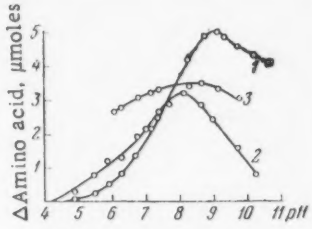

Fig. 2. pH Optimum of Glu-NH_2 transaminase. Volume of systems - 2 ml. 1) Glu-NH_2 and PhP (each 7 μmoles), incubated 1 hr at 37°; 2) Glu-NH_2 (7 μmoles) and pyruvate (14 μmoles), incubated 1 hr at 37°; 3) Glu-NH_2 and α-ketoisocaproate (each 14 μmoles), incubated 2 hr at 37°.

Effect of Thiol Poisons and Reactivators on Glutamine Transaminase Activity. The systems contained 14 μ moles glutamine and 7 μ moles of PhP in 2 ml of 0.05 M VB, pH 8.4.

Additive		ΔPhe, μ moles	Inhibition, %
inhibitor	sulfhydryl compound (reactivator)		
Control	—	3,57	—
—	Cysteine, 10^{-4} M	3,52	—
—	" $5 \cdot 10^{-4}$ M	3,56	—
—	Unithiol , 10^{-4} M	3,54	—
—	" $5 \cdot 10^{-4}$ M	3,56	—
—	GSH , 10^{-4} M	3,57	—
—	" $5 \cdot 10^{-4}$ M	3,57	—
GSSG , 10^{-3} M	—	3,14	12,0
PCMB, 10^{-5} M	—	3,21	10,1
PCMB, $5 \cdot 10^{-5}$ M	—	2,51	29,7
PCMB, 10^{-4} M	—	1,79	50,0
The same	Cysteine, 10^{-4} M	2,72	23,8
" "	" 10^{-3} M	3,45	3,4
" "	Unithiol , 10^{-3} M	3,53	1,1
" "	GSH , 10^{-4} M	3,41	4,5
" "	" $5 \cdot 10^{-4}$ M	3,57	—
" "	" 10^{-3} M	3,90	—
IAA, $5 \cdot 10^{-3}$ M	—	2,32	35,0
IAA , 10^{-2} M	—	1,53	43,0
IAA , $5 \cdot 10^{-3}$ M	Cysteine, $5 \cdot 10^{-4}$ M	2,64	26,1
The same	" 10^{-3} M	2,86	20,0
" "	Unithiol , $5 \cdot 10^{-4}$ M	2,64	26,1
" "	" 10^{-3} M	2,86	20,0
" "	GSH , $5 \cdot 10^{-4}$ M	3,04	14,9
" "	" 10^{-3} M	3,04	14,9

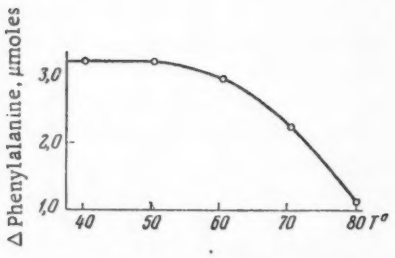

Fig. 3. Thermal inactivation of glutamine transaminase. One ml of enzyme solution containing 0.25 mg of protein was heated, 14 μ moles of glutamine and 7 μ moles of PhP in 2 ml of 0.05 M VB, pH 8.4, was added, and the systems were incubated for 1 hr at 37°.

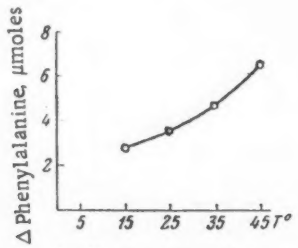

Fig. 4. Temperature dependence of the rate of transamination of glutamine (for experimental details see text).

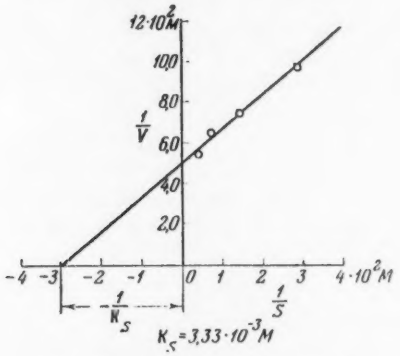

Fig. 5. Relation between rate of transamination between glutamine and phenyl phenylpyruvate ($7 \cdot 10^{-3}$ M) and glutamine concentration (from 3.5 to $28 \cdot 10^{-3}$ M). Lineweaver - Burk plot.

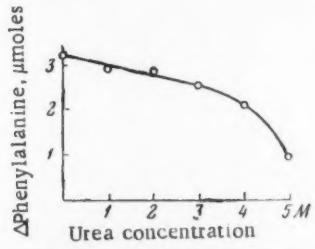

Fig. 6. Denaturalization of Glu-NH_2 Transaminase with urea.

cycloserine) on the activity of glutamine transaminase or of the "glutaminase II" enzyme system [7, 11]. We investigated the sensitivity of glutamine transaminase to thiol poisons; as is now known, enzymes for which pyridoxal phosphate acts as co-factor are highly sensitive to such agents. The action of thiol poisons on glutamine transaminase has not previously been investigated; we could find only one reference in the literature to this subject [7], reporting that iodoacetate inhibited the activity of "glutaminase II."

In our experiments glutamine transaminase was preincubated with inhibitors for 10 min at 25°. We then added to some of the systems sulfhydryl compounds, with the object of restoring activity, and continued preincubation for a further 10 min, after which substrate was added to all systems, which were incubated for 1 hr at 37°. It is evident from the table that a number of thiol poisons (PCMB, IAA, oxidized glutathione) inhibit the activity of glutamine transaminase. The inhibition was found to be reversible; it could be abolished by addition of a number of thiols (cysteine, Unithiol, reduced glutathione), which caused full or partial reactivation of the enzymatic activity.

Under the given conditions, 10^{-4} M p-chloromercuribenzoate caused 50% inhibition of transamination. Full activity was restored by adding cysteine to 10^{-3} M, GSH to $5 \cdot 10^{-4}$ M, or Unithiol (2,3-dimercaptopropane-1-sulfonate) to 10^{-3} M. Iodoacetamide ($5 \cdot 10^{-3}$ M) gave 35% inhibition, which was only partially reversed (to 20%) by cysteine or Unithiol (10^{-3} M), and to a somewhat higher degree (to 14.9%) by reduced glutathione ($5 \cdot 10^{-4}$ to 10^{-3} M).

SUMMARY

A procedure has recently been developed in this laboratory [10] for extensive purification of glutamine-α-keto acid transaminase (Glu-NH_2-transaminase) from rat liver, eliminating glutaminase, keto amidase and most contaminating transaminase activities.

The purified Glu-NH_2-transaminase has been used for the study of some physico-chemical and catalytic properties of the enzyme. The rates of transamination between Glu-NH_2 and α-keto acids (pyruvate, phenylpyruvate, α-ketoisocaproate) were studied by quantitative estimation of the new-formed amino acids on paper chromatograms. The reaction rates were practically linear for 1 ½ hr. The enzyme is relatively heat-stable, about 35% of its activity surviving 5 min heating to 80° or 2 min in the boiling water-bath. The temperature coefficient is relatively low for an enzymic reaction; with increasing incubation temperature, the rate of transamination is augmented about three-fold in the range from 15° to 45°.

pH-Optima for action of purified Glu-NH_2-transaminase are variable, depending on the NH_2-group acceptor: with pyruvate activity, maximal is at pH 8.2; with α-ketoisocaproate, at 8.7; with phenylpyruvate, at 9.1. With the latter two α-keto acids, considerable activity persists in very alkaline media (~ pH 10). The enzyme-substrate dissociation constant for Glu-NH_2, K_s, is $3.33 \cdot 10^{-3}$ M.

Glu-NH_2-transaminase is irreversibly inactivated by urea in high concentrations (in 5 M urea solution, the activity is lowered by 68%). Glu-NH_2-transaminase is strongly inhibited on preincubation with thiol poisons: 10^{-4} M p-chloromercuribenzoate (50% inhibition), $5 \cdot 10^{-3}$ M iodacetamide (35%), and less efficiently by GSSG. The activity can be restored, more or less completely, with the aid of sulfhydryl compounds (cysteine, GSH, 2,3-deimercaptopropane-1-sulfonate).

The author is deeply indebted to Prof. A. E. Braunshtein for his unfailing guidance throughout this work.

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RUSSIAN JOURNALS FREQUENTLY CITED
[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiya	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiya	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Émbriol.	Arkhiv Anatomii Gistologii i Émbriologii	Archives of Antomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiya	Biokhimiya	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiya Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Byull. Éksptl. Biol. i Med.	Byulleten Éksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Byull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Byulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Éksptl. Khirurg.	Éksperimentalnaya Khirurgiya	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiya i Toksikologiya	Pharmacology and Toxicology
Farmatsiya	Farmatsiya	Pharmacy
Fiziol. Rastenii	Fiziologiya Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenov Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariya	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiya Akademii Nauk SSSR, Seriya Biologicheskaya	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiya Tikhookeanskogo N. I. Instituta Rybnogo Khozyaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiya	Khirurgiya	Surgery
Klin. Med.	Klinicheskaya Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaya Radiologiya	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiya	Mikrobiologiya	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhyat. i Psikhogig.	Nevropatologiya, Psikhyatriya i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediya, Travmatologiya i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriya	Pediatriya	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Éndokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniya Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaya Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journal
Stomatologiya	Stomatologiya	Stomatology

* BRITISH-AMERICAN TRANSLITERATION SYSTEM.

(continued)

(continued)

Abbreviation	Journal	Translation
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriya	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiya	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiya	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiya, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoyuz. Gidrobiol. Obshchestva	Trudy Vsesoyuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoyuz. Inst. Ekspti. Med.	Trudy Vsesoyuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiya	Urologiya	Urology
Uspekhi Biokhimiya	Uspekhi Biokhimiya	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiya	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriya Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universitera, Seriya Biologii i Pochvovedeniya	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiya	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariya	Veterinariya	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniya	Voprosy Pitaniya	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaya Laboratoriya	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiya	Journal of General Biology
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The Treatment of Peptic Ulcerations by Intraarterial Procaine Infusions [Clinical Features of the Patients Under Investigation; Influence on Subjective Symptoms; Roentgenological Findings During Treatment; Acid-secreting Function of the Stomach During Treatment; Changes in the Blood Picture; Changes in the Zones of Cutaneous Sensation; State of the Cardiovascular System (Electrocardiography and Plethysmography) During Treatment.]

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